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(57) Abstract

The invention comprises a method of enhancing the immunogenicity of an envelope virus glycoprotein in a host organism. The method comprises administering to the host a composition comprising the virus envelope glycoprotein and at least one oligopeptide derived from the amino acid sequence of the envelope glycoprotein, wherein the oligopeptide contains or corresponds to virus-neutralization epitopes. The method and compositions are useful for vaccinating against viruses, such as HIV, SIV, HTLV-I, HTLV-II, or any retrovirus capable of inducing AIDS in its natural host.

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Description

INDUCTION OF PROTECTION AGAINST VIRAL INFECTION BY SYNERGY BETWEEN VIRAL PROTEINS AND VIRAL PEPTIDES.

Technical Field

This invention relates to a vaccination process, which involves the simultaneous or consecutive use of a priming antigen, in this case the glycoprotein from a virus, such as HIV, SIV or any lentivirus capable of inducing AIDS in its natural host, or from an HTLV-I or HTLV-II type retrovirus, and an amplifying composition comprised of synthetic oligopeptides, which are free or bound to a carrier molecule and in which the oligopeptides correspond to the neutralization epitopes for this same glycoprotein. This invention also relates to a composition for use in the process.

An effective vaccine composition against viruses must produce rapid neutralization of the viruses in order to prevent the viruses from possibly protecting themselves in a latent provirus form within the chromosomes of resting cell or from finding refuge in the cellular or tissue compartments where they would be beyond the reach of the immune system.

Background Art

From previous experiments conducted with both chimpanzees in the case of HIV and macaques in the case of SIV, it is clear that inoculation of virus envelope glycoprotein alone does not make it possible to obtain a fully protective immune response. In particular, the virus envelope glycoprotein does not produce a sufficient level neutralizing antibodies in order to provide protection against infection.

Accordingly, there exists a need in the art for a method of inducing a sufficient level of neutralizing antibodies against virus infection in a host susceptible to the infection by the virus. In addition, there exists a need in the art for a pharmaceutical composition for use in the method.

Disclosure of the Invention

This invention aids in fulfilling these needs in the art. An object of this invention is to reinforce the immunogenicity of at least one envelope glycoprotein of a virus by combining the glycoprotein with at least one peptide, and preferably at different times a group of peptides, derived from the sequence of the envelope glycoprotein and corresponding to virus-neutralization epitopes, i.e. corresponding to amino acid sequences involved in the production of neutralizing antibodies in the host to which they are administered.

Accordingly, this invention provides a method of enhancing the immunogenicity of an envelope glycoprotein of a virus in a host and a composition for use in this method. The method comprises administering to the host at least one envelope glycoprotein of the virus and at least one peptide derived from the amino acid sequence of the envelope glycoprotein. The peptide comprises at least one virus-neutralization epitope. The envelope glycoprotein and the peptide are administered in an amount sufficient to induce neutralizing antibodies in the host.

The invention provides a composition for enhancing the immunogenicity of an envelope glycoprotein of a determined virus, wherein the composition comprises as a combined preparation for simultaneous, separate, or sequential use:

(A) at least one envelope glycoprotein of the virus or a fragment of at least 50 amino acids of the glycoprotein and,

(B) at least one peptide derived from the amino acid sequence of the envelope glycoprotein, and wherein the peptide comprises at least one virus-neutralization epitope

and wherein the envelope glycoprotein and the peptide are administered in an amount sufficient to induce neutralizing antibodies in the host.

For the purpose of the invention, the word "composition" is intended to comprise combined preparation in which the components - in this case the envelope glycoprotein and the peptide or peptides derived from the envelope glycoprotein - can be presented in a mixture or can be presented side-by-side and therefore be applied simultaneously, separately or at intervals, to the host. For instance, the peptide(s) present in the composition can be maintained separated from other components in order to be administered sequentially to booster the immunogenic reaction which is primed with the envelope glycoprotein.

In a preferred embodiment, the invention provides a composition which comprises the above envelope glycoprotein and peptide providing the envelope glycoprotein is present in an amount sufficient for priming the induction of neutralizing antibodies in a host to which it is administered, and the at least one peptide is in an amount sufficient to enhance the induction of persistent neutralizing antibodies in the host to which it is administered.

Accordingly, the invention concerns the use of at least one of the above described peptides for enhancing the immunogenicity of an envelope glycoprotein of a virus, when this glycoprotein is administered to a host to induce neutralizing antibodies.

The composition of the invention can be used for the preparation of an immunotherapeutic drug. In this case the composition is administered to seropositive people in order to increase the level of neutralizing antibodies and accordingly to enable a control of the virus.

Methods are described by J. Salk in "4° Colloque des Cent Gardes - Retroviruses of human AIDS and related animal diseases - Ed. M. Girard, L. Valette - Foundation Merieux 1990 p. 273-278" and in "Nature 1989, vol. 327, p. 473-476."

This invention also provides a composition for vaccinating a host against infection by a virus. The composition comprises at least one envelope glycoprotein of the virus in an amount sufficient for priming vaccination in a host to which the envelope glycoprotein is administered. The composition also contains at least one peptide derived from the amino acid sequence of the envelope glycoprotein. The peptide comprises at least one virus-neutralization epitope of the glycoprotein. The composition contains the peptide an amount sufficient to enhance the induction of persistent neutralizing antibodies in the host.

The description of the invention in connection with the use as vaccine of the defined composition can also be applied to the use as immunotherapeutic drug of this composition, provided that the described means enables the enhancement of the production of neutralizing antibodies.

Peptides and envelope glycoproteins can be combined under conditions allowing them to interact by non-covalent physical combination or by covalent chemical bonding. Alternatively, and in a preferred embodiment of the invention, a priming vaccination (priming) is achieved by injections of envelope glycoprotein, with protective immunity being subsequently enhanced by the injection of immunogenic peptides corresponding to the neutralization epitopes.

Two of three immunized chimpanzees were successfully protected against virus infection and virus was suppressed in a third animal for a long period using the compositions and methods of this invention. These results demonstrate that this invention makes it possible to elicit protection against HIV-1 through immunization.

<u>Brief Description of the Drawings</u>

This invention will be more fully described by reference to the following Figures in which:

Fig. 1 depicts anti-HIV antibody level measured by ELISA (Genetic Systems Kit) in chimpanzee FUNFACE (C-339) and a control (C-519). The results are shown as serum ELISA titre (1:dilution giving positive response) versus time. Time zero

in the Figure corresponds to the day of the first booster with inactivated HIV. The animal was challenged at 70 weeks (arrow).

Fig. 2 depicts neutralizing antibody level in chimpanzees FUNFACE (dark circles) and ROBERT (open circles) in response to the injection of a KLH-BRU peptide conjugate (arrows). The animals were inoculated at 0, 3, and 19 weeks (arrows) and challenged at 24 weeks.

Fig. 3 depicts anti-HIV antibody levels measured by ELISA in chimpanzee ROBERT (C-433). The results are shown as serum ELISA titre (1:dilution giving positive response) versus time. Time zero corresponds to the day of the first antigen injection (gp160env, p27nef, p23vif, and p18gag). The animal was challenged at 84 weeks (arrow).

Fig. 4 depicts neutralization of HIV-1 BRU as a function of the serum dilution in chimpanzees JOJOTOO (499), IRA (151), and HENRY II (531) at time t0 ([.]) and at 2 weeks ([]) and 5 weeks (◆) after a third inoculation of free peptides.

Fig. 5 depicts neutralization of HIV-1 BRU (dotted curves) and HIV-1 ARV-2 (solid curves) as a function of the serum dilution in chimpanzee JOJOTOO (C-499) at time t0 ([.]) and after the third inoculation of free peptides (◆).

Fig. 6 shows total HIV-1-specific antibody titers for chimpanzees C-339 (A), C-433 (B), and C-499 (C). At the indicated times, chimpanzees were inoculated with various immunogens (see Table 1) or challenged with HIV-1. Titers are defined as the reciprocal of the highest dilution of serum that was positive using an HIV-1 ERA kit (Genetic Systems).

Fig. 7 depicts neutralizing antibody titers in serum from C-339, C-433 and C-499 during immunization with HIV-1 antigens. Titers are the reciprocal of the highest dilution of serum that gave 90% reduction in number of syncytia formed by CEM-SS cells (Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A. & Fischinger, P.J. (1987) *AIDS Res. Human Retroviruses* 3, 283-302.) when

compared to that obtained with control serum from a naive chimpanzee.

Fig. 8 shows PCR analysis of DNA from PBMC and lymph node tissue obtained 6 months after challenge of chimpanzees C-339 and C-433 with HIV-1.

(A) Ethidium bromide-stained gel of amplified HIV sequences following two rounds of PCR with nested sets of primers. The size of the HIV-specific amplified fragment is 141 base pairs.

Lane 1, 0.5 μg of OX174 DNA cleaved with *Hae*III as molecular weight markers.

Lanes 2-7, positive controls for sensitivity, each containing tenfold fewer molecules of pHXB2 cleaved with *Xba*I than the previous sample, starting with 3000 molecules in lane 2. Each sample was amplified in the presence of 1 μg DNA (the amount of DNA in 1.5 x 10^5 cells) from an uninfected control chimpanzee, C-519. One negative control sample (lane 14) was identified and used as a source of uninfected chimpanzee cellular DNA; all other samples were tested blindly. C-487 was an HIV-1 infected chimpanzee, used as a positive control.

Lanes 8-11, DNA from PBMC of C-339, C-487, C-43 and C-519, respectively.

Lanes 12-15, DNA from lymph node tissue of C-487, C-433, C-519 and C-339, respectively.

(B) Ethidium bromide-stained gel of an amplified portion of the beta-globin gene (Scharf, S.J., Horn, G.T. & Erlich, H.A. (1986) *Science* 233, 1076-1078), as an internal control. (C) Oligonucleotide hybridization of PCR-amplified sequences. PCR reaction products shown in (A) were denatured and annealed with [^{32}P]-labeled primer SK102, which anneals entirely within the amplified sequence; the products were examined following polyacrylamide gel electrophoresis and authoradiography according to Kwok and Kellogg (Kwok, S. & Kellogg, D.E. (1990) in *PCR Protocols: A Guide to Methods and Applications:* eds. Innis, M.A., Gelfand, D.H., Sninsky

J.J. & White T.J. (Academic Press, Inc., San Diego, CA) pp. 337-347).

Fig. 9 depicts immunoblot analysis of antibodies to specific HIV-1 proteins following immunization and challenge of chimpanzees C-433, C-339 and C-499. Serum samples were diluted 1:200 and tested with a commercial kit (Diagnostics Pasteur). For the samples shown, sera were collected one month prior to challenge (marked by arrow) and then at 4 week intervals. Molecular weights of HIV-1 proteins are shown for positive control serum.

Fig. 10 shows anti-gp160 ELISA titers in Rhesus monkeys treated according to the invention.

Fig. 11 shows anti-V3 BRU antibody titers in Rhesus monkeys treated according to the invention.

Fig. 12 shows the serum neutralization of cell-to-cell transmission.

Fig. 13 shows the antibody titer in chimpanzees, after cell-free HIV challenge.

Fig. 14 Schedule of immunization. Purified recombinant gp160 and PND oligopeptide (V3) were infected at the times indicated in the presence of either alum, IFA or SAF-1.

Fig. 15 Time course of antibody response in the 3 groups of monkeys as measured by ELISA. Panel A: Anti-gp160 response. Panel B: anti-V3 response. Geometric mean antibody titers in groups A (0), B ([]) and C (Δ) were computed from the data in tables 1 and 2 and plotted as a function of time of immunization.

Fig. 16 Time course of neutralizing antibody response in animals 57, 59 (group B), 61 and 64 (group C). Titers have been expressed as the reciprocal of the dilution of serum giving 50% reduction of syncitium formation.

Figs. 17 and 18 Correlation between anti-PND ELISA titers and HIV-1 neutralizing antibody titers. Panel A: titers at 5 months; panel B: titers at 7 months. 0: group A; []: group B; Δ: group C.

Best Mode for Carrying Out the Invention

Previous attempts to protect chimpanzees against HIV infection by vaccination have failed, despite the use of several different types of vaccines: synthetic peptides, live recombinant vaccinia virus (VV) expressing HIV antigens, native or recombinant gp120 or gp160 envelope antigens, and inactivated whole virus. The failure to protect a chimpanzee against an infectious HIV challenge by prior vaccination with recombinant VV followed by formalin- and betapropiolactone-inactivated whole HIV was previously reported.

This failure led to two considerations on which the present approach is based:

1 - Protection against infection with cell-free HIV probably requires high levels of neutralizing antibodies (Ab). Should the virus escape eradication by neutralizing AB or antibody dependent cellular cytoxicity (ADCC), the virus could easily remain sheltered from the immune system, either as an integrated provirus and/or by infection of cells in the bone marrow or central nervous system. Replication of the virus, even if limited, could lead to the early emergence of neutralization escape mutants. Therefore, rapid neutralization of the challenge virus may be a key to successful vaccination.

2 - Up to 1990, induction of neutralizing Ab by all the vaccines tested in chimpanzees has been at best mediocre. This may explain their failure to protect the animals against infection. To be efficacious, a vaccine, therefore, should induce higher neutralizing Ab titers than those obtained so far.

It was, therefore, sought to elicit the highest possible neutralizing Ab titers in chimpanzees through successive immunization protocols using a variety of immunogens.

One chimpanzee, C-339, was immunized initially with four injections (at 0, 1, 2 and 6 months) of 250 µg of formalin and betapropiolactone-inactivated whole HIV mixed with SAF-1 using a concentration of 1 mg threonyl MDP. The animal developed high HIV ELISA titers (1:200,000, using the ELAVIA kit from Diagnostic Pasteur with a cutoff of 0.1) and showed strong reactivity by Western blot to gp160, gp120, and gp41 _env,_ and to p55, p40, p25, and p18_gag_. Its neutralizing Ab titers reached 1:400 and 1:64, respectively, using two different neutralization assays; the first assay scored for 50% inhibition of immunofluorescent foci formation on MT4 cells, and the second one for 90% inhibition of syncytia formation on CEM-SS cells. Using a more stringent assay (100% inhibition of reverse transcriptase production in fresh human PBL), the maximum titer of neutralizing Ab was 1:160, obtained immediately after the booster injection. These titers, however, did not persist, but quickly declined to lower levels.

In an attempt to increase the neutralizing Ab titers of chimpanzee C-339, the animal was boosted repeatedly with recombinant soluble gp160_env_ purified from the supernatant of BHK-21 cell cultures infected with VV-1163, a VV-env recombinant expressing a gp160 molecule containing a deletion of the transmembrane domain and a modification by site-directed mutagenesis of the gp120/gp41 cleavage site to prevent cleavage. Vaccinia virus VV-1163 can be made using the procedures described by Kieny et al., Protein Engineering _2_:219-226 (1988). The antigen was purified by sequential

lectin and cation-exchange chromatography, then was injected I.D. at multiple sites of the chest (125-150 μg per injection) with a human dose of BCG. This was followed by 3 successive I.M. injections of the antigen formulated with SAF. ELISA and neutralizing Ab titers were followed on routinely; however, both remained unchanged during and after this course of immunizations.

Failure of the gp160<u>env</u> to enhance antibody responses was not due to lack of immunogenicity, as shown by immunizing in parallel a naive chimpanzee, C-519, which previously had not been exposed to HIV antigens. Using the same immunization protocol as for C-339, C-519 readily developed a strong anti-gp160 Ab response, and its ELISA titer reached 200,00 after two injections. Therefore, failure of C-339 to respond to the injections of gp160<u>env</u> was not due to lack of potency of the immunogen, but most likely to some unidentified, immunological block in the animal. It was reasoned that such an impairment might be by-passed by injecting the animal with only those epitopes of the gp120 molecule that were required for induction of neutralizing Ab.

It has been shown that HIV neutralizing Ab are primarily directed against the type-specific, hypervariable loop from the V3 region of gp120. Therefore, using bis-diazobenzidine, a 25-mer oligopeptide with the sequence of that loop 4-(YNTRKSIRIQRGPGRAFVTIGKIGN) from the HIV-I BRU (IIIb) strain was cross-linked to KLH. C-339 was injected with the peptide-carrier conjugate in the presence of SAF (300 μg of peptide) at 0, 3, and 19 weeks. No increase in ELISA titer was observed, but sustained neutralizing Ab titers were obtained following the second injection. The animal was challenged on week 26 (see below), together with another chimpanzee, ROBERT, C-433, that had undergone a parallel, albeit distinct, course of immunization.

Chimpanzee C-433 had been primed with VV-1139, a VV recombinant expressing the same uncleaved version of gp160<u>env</u> as VV-1163, but containing the transmembrane domain. Vaccinia virus VV-1139 can be made using the procedures

described by Kieny et al., Protein Engineering 2:219-226 (1988). Scarification was done with 2 x 10^8 PFU of the VV recombinant and was repeated at 4 and 22 weeks. The animal was then immunized with 125-150 µg each of recombinant soluble gp160, purified as described above, and recombinant p18gag, p27nef and p23vif (purified from *E. coli*) mixed with SAF. Injections were at 0, 1, 2, and 6 months, and resulted in an ELISA Ab titer of 1:400,000. Again, however, neutralizing Ab titers remained low (1:400 and 1:128, by the immunofluorescent focus and syncytia-forming assays, respectively). C-433, therefore, was injected with the same V3 peptide-KLH conjugate, according to the same immunization protocol, as C-339. The neutralizing Ab titer of C-433 was immediately boosted several fold and the animal was challenged in parallel with C-339.

The two chimpanzees were challenged using a titrated virus stock (III B stock, lot No. 40) from the National Cancer Institute (a kind gift of Larry Arthur, NCI, Frederick, MD). The stock, which contained 10^4 TCID50/ml, was diluted 1:100, and 1 ml of the dilution was injected I.V. into both of the immunized animals. To prevent unnecessary use of an animal, and in view of the fact that the virus stock had been titrated twice in chimpanzees and its infectivity for chimpanzees had been assessed regularly, no control naive chimp was used in this experiment. The chimp ID50 of this virus stock was equivalent to 4 TCID50, and in two experiments, injection of chimpanzees with 40 TCID50 resulted in the appearance of detectable virus in PBL as early as 2 weeks after injection and was followed by seroconversion at 4 weeks.

By contrast, the challenge of chimpanzees C-339 and C-433 with 100 TCID50 was not followed by detectable increases in antibody titers during the 24 weeks that have elapsed since time of challenge. In addition, C-433 has not developed anti-p25gag Ab, C-339 has not developed anti-p27nef Ab, nor have the 2 animals developed anti-p66pol Ab.

PCR tests done at 6 weeks, 12 weeks, and 24 weeks after challenge on PBL from both chimpanzees were negative, whereas the insufficiently immunized chimpanzee (C-487) that was challenged and became infected a year ago, was positive by PCR. Finally, virus has not been recovered by cocultivation of PBL from either C-339 or C-433 with human PBL, as judged by absence of RT activity after 6 weeks of culture.

It is understood that the expression "neutralization epitopes" is taken to mean, in the case of HIV-1, the major virus-neutralization epitope, such as described, among others, by Putney et al. in 1986 (Science 234:1392-1395) and by Rusche et al. in 1988 (Proc. Natl. Acad. Sci. USA 85:3198-3202), for which the sequence corresponds approximately to amino acids 296 to 331 of the HIV-1 envelope glycoprotein as described in the work of Myers et al. (Human Retroviruses and AIDS 1989, Los Alamos, Natl. Lab). Also covered by the invention are peptides corresponding to equivalent regions of different variants of HIV-1, or another retrovirus, HIV-2, HTLV-I, or HTLV-II in humans, FIV, FeLV, or another lentivirus in animals, and which correspond to the neutralization epitopes of the virus under consideration.

Also included in the scope of the invention are peptides corresponding to those known as minor neutralization epitopes, characterized by the fact that they belong to conserved regions of the envelope glycoprotein, and that they induce antibodies capable of neutralizing, at relatively low titers, several different isolates of the virus under consideration, for example several different isolates of HIV-1, or even different isolates of HIV-1, and also of HIV-2. An example of a minor epitope can be found in the work of Chanh et al. in 1986 (The EMBO Journal, 5:3065-3071) and in that of Evans et al. in 1989 (Nature 339:385-388), or Almond et al. in "Retroviruses of human AIDS and related animal disease," M. Girard and L. Valette, Foundation Marcel Merieux, Lyon, 1990, in press).

Immunogenic peptides of major and minor neutralization epitopes are preferably mixed with each other to ensure the

greatest possible protection. They can be administered in the free state, not coupled to a carrier molecule. They can also be combined with a sequence of amino acids having one or preferably several T-epitopes from one or several structural or non-structural proteins of the same retrovirus or a retrovirus immunologically cross-reactive with the former, particularly such as described in French patent application of Girard-Gluckman-Bahraoui, No. 89.11044 of August 18, 1989.

In one particularly preferred embodiment of the invention, immunogenic peptides corresponding to neutralization epitopes are chemically coupled to sequences of amino acids corresponding to T-epitopes. In another case, the peptides are coupled to a carrier molecule which bears the desired T-epitopes, by allowing them to react, for example, with a bifunctional reagent or any other coupling agent desired.

As a carrier molecule, any protein coded for by the viral genome can be used (in the case of HIV, the proteins produced by tat, rev, vif, pol, vpr, vpx, vpu, gag, env, or net genes), or other (protein-type) molecules, such as HBs antigen, HBc antigen, tetanus toxoid, hemocyanin, human albumin, or polypeptides (for example polylysine) or appropriate lipopeptides.

In a particular embodiment of the invention in which the envelope glycoprotein molecules and major and minor neutralizing peptides (either free or bound to carrier molecules) are combined in the same vaccine preparation, the priming effect of the envelope glycoproteins appears after the first one or few injections of vaccine, and the amplification effect due to peptides immediately afterward.

Thus, an object of the invention is to use a first antigen, in this case the several envelope glycoproteins of each of the retrovirus serotypes under consideration, which has the effect of priming the response of the immune system; and a second antigen, in this case the synthetic peptides corresponding to major and also possibly minor neutralization epitopes of the different serotypes of the virus under

consideration, for vaccination (preferably consecutively, but in a mixture, if necessary) with the purpose of amplifying and consolidating the initial response, particularly through induction of long-lasting, high-titer neutralizing antibodies. This invention makes it possible to induce immunity that persists as long as about six months and even as long as one year or more.

The glycoproteins used to prime the response of the immune system are preferably whole molecules as obtained before possible cleavage. Thus, in the case of HIV-1, gp160 is preferable to gp120, and the same is true for other retroviruses. This allows anti-gp41 antibodies in particular to be induced, which is a favorable sign in virus carriers (Klasse et al., Proc. Natl. Acad. Sci. USA, 85:5225-5229).

The peptides constituting the "amplifier" can be free or physically bound (especially by hydrophobic bonding) or chemically bound (especially by covalent bonding) to carrier molecules. They can also be associated with other peptides corresponding to T-epitopes, or even to peptides, lipopeptides, glycopeptides, aliphatic chains, fatty acids, or any combination of these capable of stimulating the immune system and/or specifically targeting the "amplifier" peptides to antigen-presenting cells.

From this point of view, a particularly advantageous presentation of peptides corresponding to HIV neutralization epitopes is to bind them, preferably by covalent chemical bonding, to an aliphatic sequence, particularly as described in 1989 by Deres et al. (Nature 342:561-564). The amplifying peptides presented in this way can induce not only a B-cell response, but also a CTL $CD8^+$ response, restricted HLA Class I, as described by Takanashi et al. in 1988 (Proc. Natl. Acad. Sci. USA 85:3105-3109).

When the virus has a high degree of antigenic variability, as in the case of HIV-1 and HIV-2, it is necessary to use as priming antigen not just one, but several envelope glycoproteins with different sequences, each sequence corresponding to an isolate or group of isolates of

the virus under consideration, so as to obtain as many priming phenomena as desired, since each is specific for a single isolate or group of isolates. In this case, it is understood that the amplifying peptides are composed of the mixture of neutralization peptides of each of the isolates under consideration, as indicated below.

A preparation of HIV-1 amplifier peptides according the invention is characterized by the fact that it contain at least one of the sequences or one part of the sequences described below in one letter amino acid code:

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C-TRPNNNTRKR IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKK IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRGS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IYI--GPGRA FHTTGRIIGD -IRKAH-C
C-TRPYNNVRRS LSI--GPGRA FRTRE-IIGI -IRQAH-C
C-TRPGNNTRRG IHF--GPGQA LYTTGIV-GD -IRRAY-C
C-ARPYQNTRQR TPI--GLGQS LYTTRSR-SI -IGQAH-C
C-TRPNNNTRKS ITK--GPGRV IYATGQIIGD -IRKAH-C
C-TRPNNNTRKR ITM--GPGRV YYTTGQIIGD -IRRAH-C
C-TRPGSDKRQS TPI--GLGQA LYTTRGRTKI -IGQAH-C
C-TRPGSDKKIR QSIRIGPGKV FYAKGG---I -TGQAH-C
C-TRPNNNTKKG IAI--GPGRT LYAREKIIGD -IRQAH-C
C-TRPNNHTRKR VTL--GPGRV WYTTGEILGN -IRQAH-C
C-TRPGNNTRRG SHF--GPGQA LYTTGIVGDI -RRAY-C
C-TRPDNKITSRQ-TPI-GLGQA LYTTRIKGDI -RQAY-C
C-TRPNNNVRRR-HIHI-GPGRA FYTGEIRNI  -RQAH-C
C-TRPYKNTRQS-TPI--GLGQA LYTTRTKSI  -GQAH-C
C-TRPNNNTTRS-IHI--GPGRA FYATGDIIGTIRQAH-C
C-TRPNYNKRKR-IHI--GPGRA FYTTKNIIGDIRQAH-C
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The production of the amplifying molecules of the invention by using a sequence containing at least one neutralization epitope and particularly one of those from the list above and one carrier sequence having at least one T-epitope, may be achieved by binding these sequences or by physical combination in the same composition.

To be fully effective, priming and amplifying antigens must be enhanced, for example and preferably by lipid adjuvants, such as derivatives of muramyl dipeptide in lipid emulsions, or incomplete Freund's adjuvant.

The priming and amplifying antigens are preferably administered intramuscularly to a host, such as a primate, and especially a human. Following are typical immunization schedules that can be employed for gp160 and peptides of HIV

gp160 (months)	Peptides (months)
0, 1, (2), 6	12, 13
0, 1, 2, 12	13, 14
0, 1, 2, 12	1, 2, (12)

It will be understood that these immunization schedules are merely representative and that the schedules can be varied to obtain the optimum response in the host. Similarly, the amounts of the priming and amplifying antigens can be varied. For example, about 150 μg of gp160 in Syntex SAF-1 adjuvant can be administered as indicated, followed by administration of the peptides in amounts of typically 100 μg of each peptide.

Finally, the relative proportions of the peptides involved can vary according to the desired final proportions of each peptide in the final preparation. In particular, these proportions will be adjusted as a function of the immunogenicity of each peptide and the number of functional groups carried by each one, which are capable of entering into the conjugation reaction with complementary functional groups, at least when these peptides are coupled to a carrier molecule.

In a particular application of the invention, the injection of amplifying peptides is replaced by the administration of particles, virus, or bacteria, which are recombinants expressing the neutralization epitope of the virus under consideration on their surface and/or during their multiplication and in this way are capable of inducing neutralizing antibodies against said retrovirus: HBc antigen

particles; HBs antigen particles; bacteria expressing the neutralization epitope in surface or cytoplasmic proteins, such as, for example, the lamB receptor; picorna virus chimeras, such as, for example, poliovirus-HIV chimeras; poxvirus recombinants; adenovirus recombinants or adenovirus chimeras, etc. Depending on the live vector selected for the presentation of the neutralization epitope, this administration can be carried out in the form of live vaccine administered orally (for example, chimeras constructed from Sabin poliovirus strains or from human adenoviruses, or from attenuated strains of Salmonella, Shigella, or other enterobacteria, or from any organism, virus, yeast, bacteria capable of inducing an immune response after oral administration) or in the form of live vaccine administered by the parenteral route (for example, recombinant poxvirus) or even in the form of inactivated vaccine by the parenteral route (for example, chimeras constructed from the Mahoney strain of poliovirus, or inert particles of HBsAg or HBcAg).

In another particular embodiment of the invention, the antigen (envelope glycoprotein), which is injected for the priming of the vaccination, i.e., the envelope glycoprotein of the virus, is presented under the form of particles such ISCOM (Immune Stimulating Complex, comprising an association of an antigenic protein with a glycoside Quil A) or liposomes.

The priming antigen and/or the peptide can be also associated with live recombinant microorganisms, such as viruses or bacteria (for instance the poxvirus or BCG: Bacile de Calmette Gerin) or any live vaccine modified to express the envelope glycoprotein or the peptide derived therefrom.

The envelope glycoprotein and/or the peptide derived therefrom can also be presented by inactivated particles, for instance viral particles, such as the HIV virus or a part of this virus, or particles without genome. Such particles without genome have been described to produce vaccine by Haffar O. et al., Journal of Virology, 64:2653-2659 (1990) These particles can be called HIV-like particles in the case

of HIV virus: for the purpose of the invention they do not contain the complete HIV genome, but they enable the exposition at their surface of the virus components of the composition of the invention.

In another embodiment of the invention, the envelope glycoprotein antigen is combined in a mixture with other antigens. For instance, when the priming antigen is the HIV envelope glycoprotein, one or several antigens, such as gag net, vif, pol, GPG or GLG antigens, can be combined with it, as they can be combined with the peptides of the composition

The invention also comprises the compositions above described, wherein the env glycoprotein is replaced by or associated with a fragment thereof. This fragment has advantageously more than 50 amino acids and is characterize in that it has the immunogenic properties of the glycoprotein in the context of the invention.

The invention also concerns monoclonal or polyclonal antibodies, which recognize the glycoprotein and/or peptides of the composition. These antibodies can be associated in a mixture and used, for instance, for serotherapeutic purposes.

<u>EXAMPLE 1</u>: Immunization of a chimpanzee with HIV-1 BRU and the glycoprotein of this isolate; amplification of the response with a BRU env oligopeptide coupled to KLH.

Chimpanzee 339 (FUNFACE) was first immunized with three injections at one month intervals of 250 µg of purified HIV-1 BRU virus, inactivated by treatment with 0.025 percent formalin for 48 hours at 30°C and 0.025 percent betapropiolactone for 30 minutes at 37°C, combined with Syntex adjuvant containing 1 mg/ml threonyl-MDP in an emulsion of 5 percent squalane and 2.5 percent pluronic polymer. These injections were followed by a first booster at 7 months and a second booster one year later.

The animal then received five injections of BRU virus envelope glycoprotein (gp160) purified from supernatant of BHK-21 cell cultures infected with a vaccinia virus recombinant (strain VVenv 1163) having a genome for which

genetic recombination techniques were used to insert the sequences of HIV-1 BRU coding for gp160env modified through oligonucleotide site-directed mutagenesis to eliminate the sequences involved in gp120/gp41 cleavage and from which the transmembrane hydrophobic zone was deleted, as described in Kieny et al. in 1988 (Prot. Engineering 2:219-226). The purified protein was used in an amount of 125-150 µg per intramuscular injection in the presence of Syntex adjuvant. To prepare the glycoprotein, the culture medium of BHK cells infected with VV-1163 was concentrated by precipitation with ammonium sulfate, then with trichloracetic acid, and the glycoprotein was then purified by three successive runs of affinity chromatography over lentil lectin, ion exchange over cation-exchange resin, and high-performance liquid chromatography (HPLC). The recombinant gp160 obtained in this way is 95 percent pure. It is recognized by monoclonal antibodies specific of the gp160 of HIV-1 and particularly by neutralizing antibodies 110-4 specific for the major neutralization epitope of the BRU isolate. Moreover, it shows a strong affinity for the CD4 receptor of T4 lymphocytes.

The level of antibodies induced in response to injections of inactivated virus (ELISA determination: 1/200,000 with the Diagnostics Pasteur ELAVIA kit; neutralizing titer 1/400 by measurement of 50% inhibition of the formation of immunofluorescence foci; 1/64 by measurement of 90% inhibition of syncytia formation in CEM-SS cells), was not changed appreciably by the injection of gp160.

The animal was given 300 µg of preparation of synthetic peptide having the sequence Y N T R K S I R I Q R G P G R A F V T I G K I G N corresponding to the neutralization epitope of the BRU isolate, the tyrosine residue (Y) being coupled to hemocyanine (KLH) with bis(diazobenzidine) and combined with Syntex adjuvant. The injection was repeated once three weeks later, then a second time at 19 weeks.

These injections did not result in any increase in antibody titers measured by ELISA (Figure 1), but they did

result in a marked increase in neutralizing antibodies, as can be seen in Table 1 and Figure 2, as measured by three different antibody titration methods.

Table 1

Induction of neutralizing antibodies in the chimpanzee FUNFACE (C-339)

Date after 1st injection (weeks)	Level of neutralizing antibodies measured by method		
	A	B	C
0	0	32	100
3	100		150
8	1600	128-256	800

A: 90% inhibition of syncytia in MT4 cells

B: 90% inhibition of syncytia in CEM-SS cells

C: 75% inhibition of immunofluorescence in H9 cells

FUNFACE was then challenged at 26 weeks, by administering an intravenous injection of 1 ml of a 1:100 dilution, or 100 TCID50 of an HIV-1 stock titrating 10^4TCID50/ml, kindly provided by Larry Arthur (NCI, Frederick). This stock 040 was titered on two occasions in the chimpanzee, which allowed Arthur et al. to determine that its ID50 for the chimpanzees was 4 TCID50. The injection of 40 TCID50 of this stock in unimmunized chimpanzees resulted in the appearance of detectable virus in the lymphocytes of the animal starting two weeks after injection and was followed by anti-HIV seroconversion within four weeks, as observed in the two samples, and as published by Arthur et al. in 1989 (J. Virol.).

The chimpanzee FUNFACE demonstrated apparently total protection against infection with 100 TCID50 of the stock 040 virus, because at up to six months after the challenge injection, no virus was detected in his lymphocytes (as

measured either by gene amplification with pol and gag probes, or by coculture with human lymphocytes and assay of reverse transcriptase in 100,000 x g pellets obtained from culture supernatants) and at six months, there was no anti-HIV anamnestic response as measured by ELISA or by Western blot (Table 2) and no anti-nef antibody detectable by Western blot.

Table 2

Fate of anti-gp160 and anti-major BRU neutralization epitope antibodies after challenge injection of FUNFACE

	ELISA titer on date indicated				
Antigen	day of challenge	+1 month	+2 months	+3 months	+4 months
gp160	179,000	127,000	89,000	44,000	18,000
BRU peptide	6,000	3,000	2,500	1,000	1,000

EXAMPLE 2: **Immunization of a chimpanzee with recombinant antigens env, gag, net, and vif of HIV-1; amplification of the response by a BRU env oligopeptide coupled to KLH.**

Chimpanzee 433 (ROBERT) was first primed with three consecutive scarifications of 2×10^8 PFU of a recombinant vaccinia virus (VVenv 1139) expressing the gp160env of HIV-1 BRU, then by the intravenous administration of his own lymphocytes which previously had been infected in vitro by the recombinant virus VVenv 1139 and fixed in formaldehyde. The animal then received three consecutive intramuscular injections at one month intervals, then three boosters at 33, 38, and 40 weeks and a last booster at 66 weeks consisting of a mixture of 125-150 µg of each of the following antigens combined with Syntex adjuvant: gp160env, purified as described in Example 1 above, and the proteins p18gag, p27nef, and p23vif expressed in E. coli and purified as described in French patent application No. 89.11044 of

August 18, 1989. Finally, ROBERT received the same BRU peptide coupled to KLH and combined with Syntex adjuvant on the same inoculation schedule as FUNFACE did in the previous example.

Injections of the peptide-KLH conjugate did not result in any increase in antibody levels as measured by ELISA (Fig. 3), but did result in a marked increase in neutralizing antibodies, as can be seen in Fig. 2 and in Table 3. The neutralizing antibodies were also measured using three different methods:

Table 3

Induction of neutralizing antibodies in the chimpanzee ROBERT (C-433)

Date after 1st injection. (weeks)	Level of neutralizing antibodies measured by method		
	A	B	C
0	200	64	200
3	200		200
8	>800	256-512	>1600

A: 90% inhibition of syncytia in MT4 cells

B: 90% inhibition of syncytia in CEM-SS cells

C: 75% inhibition of immunofluorescence in H9 cells

Robert was then challenged in parallel with FUNFACE, by the intravenous inoculation of 100 TCID50 of the same stock 040 of HIV-1 virus from NCI as in the previous example. Here again, total protection against infection appears to have been obtained as judging from the absence of virus in the animal's lymphocytes and the negativity of the PCR six months after challenge and by the absence of anti-p25<u>gag</u> and anti-p27<u>nef</u> antibodies, as well as the absence of anamnestic anti-HIV response as measured by ELISA or by Western blot six months after challenge. Table 4 shows the same absence of

anamnestic effect on the anti-gp160 and anti-BRU neutralization epitope.

Table 4

Fate of anti-gp160 and anti-major BRU neutralization epitope antibodies after challenge injection of ROBERT

	ELISA titer on date indicated				
Antigen	day of challenge	+1 month	+2 months	+3 months	+4 month
gp160	545,000	421,000	200,000	95,000	32,000
BRU peptide	9,000	6,000	3,000	3,000	4,000

EXAMPLE 3: Immunization of a chimpanzee with gp160env and p18gag of HIV-1 antigens; amplification with HIV-1 env peptides not coupled to a carrier molecule.

Three chimpanzees were used in this experiment: the chimpanzees JOJOTOO (499), IRA (151) and HENRY II (531).

The first, JOJOTOO, received three injections, at one month intervals, of 120-150 μg of gp160env and p18gag, purified as described above, and mixed with Syntex adjuvant. This first series of injections was followed by three boosters of the same antigen given at weeks 33, 38, and 40, and a final booster at 14 months. These injections resulted in the appearance of a high antibody level detectable by Western blot and by ELISA starting immediately after the first three injections, although the level of neutralizing antibodies was relatively low, as described below.

The second chimpanzee, IRA, was immunized with 10^8 PFU of each of the four recombinant vaccinia virus stocks expressing, respectively, gp160env, p55gag, p27nef, and p23vif of HIV-1 BRU. These inoculations given by the intradermal route, did not lead to the appearance of any neutralizing antibody, but a barely significant level

(≤1:200) of antibody was detectable by Western blot or by ELISA. Chimpanzee IRA was then rested for two years.

The fourth chimpanzee, HENRY II, was naive in regard to contact with HIV or SIV antigens before the day of the experiment.

On that day the three animals described above were injected intramuscularly with a cocktail composed of 21 synthetic peptides, corresponding to the 21 sequences of the major neutralization epitope (loop V3) of HIV-1 published in Myers et al. (1989), in the amount of 50 μg per peptide, in the presence of Syntex adjuvant. Each of the peptides had a cysteine at the N-terminal position and another at the C-terminal, and thus represented the entire V-3 loop of a given isolate (amino acids 296-331 of the BRU isolate and corresponding amino acids according to the alignment of Myers et al. (1989)). The animals were reinjected with the same mixture, respectively, 1 and 2 months after the first injection. This immunization with the mixture of peptides (1.05 mg per injection) was followed in JOJOTOO with a significant anamnestic response directed against the gp160 of the BRU isolate and against its major neutralization epitope, as measured by ELISA and by using purified gp160 BRU or BRU peptide as antigen (Tables 5 and 6).

Table 5

Induction of anti-gp160 BRU antibodies in response to the injection of a cocktail of free peptides corresponding to 21 sequences of the HIV-1 neutralization epitope (ELISA titer: anti-gp160 BRU)

	Time			
Chimpanzee	1st injection (time 0)	2nd injection (1 month)	3rd injection (2 months)	4th injection (3 months)
JOJOTOO (499)	300,000	450,000	2,500,000	700,000
IRA (151)	Negative	ND	13,000	7,000
HENRY II (531)	Negative	ND	Negative	Negative

ND: not determined

Table 6

Induction of BRU anti-neutralization epitope antibodies in response to the injection of a cocktail containing 21 peptides (ELISA anti-BRU titer)

	Time			
Chimpanzee	1st injection (time 0)	2nd injection (1 month)	3rd injection (2 months)	4th injection (3 months)
JOJOTOO (499)	6,000	10,000	380,000	200,000
IRA (151)	Negative	ND	4,000	2,000
HENRY II (531)	Negative	ND	Negative	Negative

ND: not determined

The titers obtained in IRA remained very low, and they were completely negative in HENRY II. These results clearly illustrate the priming effect on the immune response resulting from pre-immunization with gp160.

The increase in the anti-peptide and anti-gp160 titer in JOJOTOO was, however, not accompanied by a marked increase in the anti-HIV ELISA titer, as can be seen (Table 7) by using a commercial diagnostic kit (ELAVIA Diagnostics Pasteur)

Table 7

Anti-HIV antibody level as measured by ELAVIA

	Date		
Chimpanzee	Time 0 1st injection	2 months 2nd injection	5 months 3rd injection
JOJOTOO (499)	1,000,000	1,600,000	400,000
IRA (151)	Negative	800	100
HENRY II (531)	Negative	200	Negative

In contrast, the injections of the mixture of synthetic peptides corresponding to neutralization epitopes of the 21 isolates of HIV-1 were followed by a very clear increase in the level of antibodies neutralizing the BRU isolate, as shown in Table 8 and Figure 4. It is remarkable that this increase was seen only in JOJOTOO, but not in IRA nor in HENRY II, demonstrating the specificity of the priming effect of pre-immunization with gp160 (Figure 4).

JOJOTOO's neutralizing antibody response is, moreover, specific for the BRU isolate, as can be seen in Figure 5: his serum does not neutralize the SF2 isolate (ARV-2), but only neutralizes the BRU isolate (HTLV-3=LAV1).

Table 8

Level of neutralizing antibodies induced by three injections of a mixture of peptides corresponding to the 21 known sequences of the major neutralization epitope of HIV-1: 75% neutralizing titer measured on CEM-T4 cells (Method C in Table 1).

Time	
1 month before the first injection	+1 month after the third injection
250	2,500

* * *

Follow-Up Experimental Results

The most stringent test for efficacy of experimental vaccines against the human immunodeficiency virus type 1 (HIV-1) is protection of chimpanzees from infection following live virus challenge. In the study reported here, sustained high titers of neutralizing antibodies were elicited in three chimpanzees after sequential injections of different HIV-1_{BRU} antigen preparations that included whole inactivated virus or purified recombinant proteins, followed by synthetic peptides identical to the major HIV-1 neutralizing epitope, V3. The animals were challenged intravenously with 40 chimpanzee infectious doses (equivalent to 100 50%-tissue culture infectious doses "TCID") of a stock of HIV-$1_{HTLV-IIIB}$. After 6 months of follow-up, all three animals appeared uninfected by serologic and virologic criteria, including PRC analysis and failure to isolate virus from peripheral blood lymphocytes, bone marrow and lymph node tissue. Of two chimpanzees monitored for 1 year, virus was isolated initially from one animal at 32 weeks, but the second and third chimpanzees were virus negative by all assays through 12 months. The third animal has remained virus negative through 7 months of follow-up. These results indicate that it is possible to elicit protection against, or significantly delay infection of, HIV-1 by immunization, thus laying the foundation for development of an HIV-1 vaccine.

Materials and Methods

Animals. Animals used in this study were adult male chimpanzees that had been used previously in hepatitis A, B and non-A and non-B experiments. The chimpanzees were maintained at LEMSIP, New York University Medical Center, in biosafety level 3 facilities. All experimental procedures were done according to institutional guidelines for containment of infectious diseases and for humane care and handling of primates (Moor-Jankowski, J. & Mahoney, C.J. (1989) *J. Med. Primatol.* 18, 1-26).

Immunogens. Sucrose gradient-purified whole HIV was inactivated by incubation with 0.025% beta-propiolactone, followed by 0.025% formalin, and was shown not to contain infectious virus by failure to isolate virus from peripheral blood mononuclear cells (PBMC) of immunized chimpanzees (Girard, M., Kieny, M.P., Gluckman, J.C., Barre-Sinoussi, F., Montagnier, L. & Fultz, P. (1990) in *Vaccines for Sexually Transmitted Diseases eds. Meheus*, A. & Spier, R. (Butterworth Co., Ltd., London), pp. 227-237). Recombinant gp160env was purified from the culture medium of BHK21 cells infected with VV-1163, a recombinant vaccinia virus expressing the gp160env gene modified by site-directed mutagenesis to destroy the gp120/41 cleavage site and to remove the anchor domain of gp41 (Kieny, M.P., Lathe R., Riviere, Y., Dolt, K., Schmitt, D., Girard, M., Montagnier, L. & Lecocq. J.P. (1988) *Prot. Engineering* 2, 219-226; and Schmidt, D., Dezutter-Dambuyant, C., Hanau, D., Schmitt, D.A., Kolbe, H.V.J., Kieny, M.P., Cazenave, J.P. & Thivolet, J. (1989) *Comptes Rendus Acad. Sci. Paris*, 308(III), 269-275). Where indicated, the antigen was mixed with recombinant p18*gag*, p27*nef* and p23*vif* antigens that were purified from E. coli pTG2153, pTG1166 and pTG1149, respectively, as described (Guy, B., Riviere, Y., Dott, K. Regnault, A. & Kieny, M.P. (1990) *Virology* 176, 413-425; and Kolbe, H.V., Jaeger, F., Lepage, P., Roitsch, C., Lacaud, G., Kieny, M.P., Sabatie, J., Brown, S.W. & Lecocq, J.P. (1989 *J. Chromatography* 476, 99-112). Before each immunization, inactivated whole HIV (250 µg vital protein) or the purified

recombinant proteins (125-150 μg each per dose) were mixed with the adjuvant SAF-1 (Allison, A.C. & Byars, N.E. (1986) *J. Immunol. Methods* 95, 157-168), and 2 ml of the mixtures were injected intramuscularly (IM).

An aliquot (19.8 mg) of a 25-amino acid peptide, with the sequence Y-NTRKSIRIQRGPGRAFVTIGKIGN (Putney, S.D., Matthews, T.J., Robey, W.G., Lynn, D.L., Robert-Guroff, M., Mueller, W.T., Langlois, A.L., Ghrayeb, J., Petteway, S.R., Weinhold, K.J., Fischinger, P.J., Wong-Staal, F., Gallo, R.C. & Bolognesi, D.P. (1986) *Science* 234, 1392-1395; Rusche, J.R., Kavaherian, K., McDanal, C., Petro, J., Lynn, D.L., Grimaila, R., Langlois, A., Gallo, R.C., Arthur, L.O., Fischinger, P.J., Bolognesi, D.P., Putney, S.D. & Matthews, T.J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 3198-3202; and LaRosa, G.J., Davide, J.P., Weinhold, K., Waterbury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.J., Dressman, G.R. Boswell, R.N., Shadduck, P., Holley, L.H., Karplus, M., Bolognesi, D.P., Matthews, T.J. Emini, E.A. & Putney, S.D. (1990) *Science* 249 932-935) was treated first with citraconic acid and then was coupled to 19.3 mg keyhole limpet hemocyanin (KLH) by N-terminal tyrosyl linkage using bis-diazobenzidine (pH 9.0). After the block on amino groups was removed, the peptide-KLH conjugate was dialyzed for 24 hours against PBS to remove excess free peptide. After formulation with SAF-1, immunizations with the V3 peptide-KLH conjugate (300 μg peptide per dose) were done by the IM route.

Challenge Virus. The challenge inoculum was from a stock of HIV-1 strain HTLV-IIIB (obtained from L. Arthur), which had been titrated in chimpanzees and used in other HIV vaccine challenge studies (Arthur, L.O., Bess, J.W., Waters, D.J., Pyle, S.W., Kelliher, J.C., Nara, P.L., Krohn, K., Robey, W.G., Langlois, A.J., Gallo, R.C. & Fischinger, P.J. (1989) *J. Virol.* 63,5046-5053; and Berman, P.W., Gregory, T.J., Riddle, L., Nakamura, G.R., Champe, M.A., Porter, J.P., Wurm, F.M., Hershberg, R.D., Cobb, E.K. & Eichberg, J.W. (1990) *Nature (London)* 345, 622-625). The infectivity titer of this HIV-1 stock is considered to be 10^4 $TCID_{50}$ per ml and

4×10^3 infectious units per ml for chimpanzees. The chimpanzees were challenged IV with 1 ml of a 1:100 dilution. Aliquots of these same 1:100 dilutions were titrated in quadruplicate by twofold serial dilution and infection of 1×10^5 H9 cells in 96-well microtiter plates. After incubation for 6 days, infection was scored by immunofluorescence assay. By this method, the challenge inoculum had a titer of greater than 64 immunofluorescent focus-forming units (end-point not reached) for the first aliquot and 170 for the second.

Neutralization Assay. Neutralization activity in serum samples from immunized chimpanzees was determined by inhibition of syncytia formation in CEM-SS cells, as described (Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A. & Fischinger, P.J. (1987) *AIDS Res. Human Retroviruses* 3, 283-302), or inhibition of immunofluorescent foci in H9 cells.

Virus Isolation. PBMC or bone marrow cells (obtained as aspirates) from immunized and challenged chimpanzees were cultured with normal human PBMC, as described (Fultz, P.N., McClure, H.M., Swenson, R.B., McGrath, C.R., Brodie, A., Getchell, J.P., Jensen, F.C., Anderson, D.C., Broderson, J.R. & Francis, D.P. (1986) *J. Virol.*, 58, 116-124). In some experiments, CD^+4-enriched lymphocytes were obtained from chimpanzee PBMC by separation with magnetic beads to which were attached monoclonal antibodies specific for the CD8 cell-surface antigen (Dynabeads, Robbins Scientific). The CD+4-enriched cells were stimulated 2 days with concanavalin A (10 μg/ml) before being cultured alone or cocultured with phytohemagglutinin (PHA)-stimulated normal human PBMC in RPMI-1640 medium with 10% fetal bovine serum, glutamine, gentamicin and recombinant interleukin-2 (8 units/ml; Boehringer Mannheim). Lymph nods tissue obtained by biopsy was minced with scissors and cultured with human PBMC. All cultures were maintained and monitored for reverse transcriptase activity for 6 weeks before being discarded.

Polymerase Chain Reaction (PCR). Both single- and double-round (nested) PCR were performed periodically with

PBMC or lymph node cells from challenged chimpanzees. Single-round PCR was as described (Laure, F., Rouzioux, C., Veber, F., Jacomet C., Courgnaud, V., Blanche, S., Burgard, M., Griscelli, C. & Brechot, C. (1988) *Lancet* 2, 538-541). Briefly, 2 μg DNA were used with 2 units Taq-1 DNA polymerase for 40 cycles at 94°C, 55°C, and 72°C (1 min. each). Two primer pairs were used: one corresponded to nucleotides 2393-2417 and 2675-2700, encoded by the *pol* gene, and the other corresponded to nucleotides 5367-5385 and 5694-5711, encoded by the *tat* gene. To show specificity of the PCR, amplified DNA fragments were hybridized with [^{32}P]-labeled internal *pol* and *tat* gene probes. The positive control consisted of DNA from the 8E5 cell line persistently infected with LAV-1. For nested PCR, the primers for the first round of PCR, performed as described (Mullis, K.B. & Faloona, F.A. (1987) *Methods Enzymol.* 155, 335-350) were: 5'-GCTTCTAGATAATACAGTAGCAACCCTCTATTG-3', corresponding to a 3-base clamp sequence, an *XbaI* restriction site and nucleotides 1025-1048 of the HXB2 genome, and: 5'-GTCGGCCTTAAAGGCCCTGGGGCTTGTTCCATCTATC-3', corresponding to a 3-base clamp sequence, a *NotI* restriction site and nucleotides 5573-5553 of the HXB2 genome. From the first round, 2.5 μl of the product was reamplified with primers SK145 and SK150 (Kwok, S. & Kellogg, D.E. (1990) in *PCR Protocols: A Guide to Methods and Applications*: eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White T.J. (Academic Press, Inc., San Diego, CA) pp. 337-347), over a region from nucleotides 1366 to 1507 on the HXB2 genome.

Immunization Regimens (Table 9)

TABLE 9. Immunization regimens of chimpanzees with various HIV-1 antigens

Animal	Recombinant VV-1139	Inactivated HIV	Recombinant antigens gp160	gag	nef	vif	V3 peptide
C-433	+	+	+	+	+	+	+
C-339	-	+	+	-	-	-	+
C-499	-	-	+	+	-	-	+

For C-433 and C-339, times of immunizations and virus challenge were calculated from the time that C-433 received its first immunization with VV-1139, which is considered week 0. Chimpanzee C-433 was first immunized with a recombinant vaccinia virus, VV-1139, that expresses a non-cleavable version of the HIV-1_{BRU} gp160*env* antigen (Kieny, M.P., Lathe R., Riviere, Y., Dott, K., Schmitt, D., Girard, M., Montagnier, L. & Lecocq, J.P. (1988) *Prot. Engineering* 2, 219-226). VV-1139 was administered on weeks 0, 8 and 21 by scarification on the upper back with a two-pronged needle (2×10^8 PFU per inoculum). At week 27, PBMC from C-433 were stimulated with PHA, cultured in medium containing IL-2 and then infected with VV-1139 at a multiplicity of infection of 7. Following culture for an additional 16 hours, the PBMC were fixed with 0.8% paraformaldehyde and reinjected into C-433 by the IV route (Zagury, D., Bernard, J., Cheynier, R., Desportes, I., Leonard, R., Fouchard, M., Reveil, B., Ittele, F.D., Lurhama, Z., Mbayo, K., Wane, J., Salaun, J.J., Goussard, B., Dechazal, L., Burny, A., Nara, P. & Gallo, R.C. (1988) *Nature (London)* 322, 728-731). At weeks 48, 54, 58, 81, 86, 88, 114 and 124, C-433 was inoculated IM with mixtures of purified gp160*env*, p18*gag*, p27*nef* and p23*vif* (125-250 µg each per dose) formulated with SAF-1.

Chimpanzee C-339 was first immunized on week 33 by IM injection of inactivated HIV (125 µg viral protein) mixed

with SAF-1 (1 mg threonyl muramyl dipeptide), followed by booster inoculations on weeks 37, 41, 62 and 124. C-339 was then inoculated with purified gp160*env* only (125 μg per dose) on weeks 66, 74, 81, 85 and 87. The V3 peptide (300 μg peptide per dose) was administered IM on weeks 105, 108 and 126.

C-339 and C-433 were challenged on week 131 with 100 $TCID_{50}$ of $HIV\text{-}1_{HTLV\text{-}IIIB}$. C-449 was inoculated IM with a mixture of gp160*env*, p18*gag* and SAF-1 on weeks 0, 6, 10, 33, 38, 66 and 76. (Note: week 0 for C-499 corresponds to week 48 for C-433 and C-339.) A mixture of 21 free V3 peptides (100 μg each per dose) was administered IM with SAF-1 on weeks 79, 83, 87 and 102. C-499 and C-087, a naive control, were challenged on week 106 and 100 $TCID_{50}$ of $HIV_{HTLV\text{-}IIIB}$.

<u>Results</u>

Immunization of chimpanzee C-339 with formalin- and beta-propiolactone-inactivated whole HIV mixed with the adjuvant SAF-1 resulted in high titers of antibodies to *gag*- and *env*-encoded proteins, as measured by enzyme immunoassay (EIA), a low neutralizing antibody response, and no detectable cell-mediated immune response. In an effort to enhance immune responses, C-339 was immunized with purified recombinant gp160*env*. Following one intradermal inoculation of gp160*env* with BCG in multiple sites on the chest, C-339 was given four successive IM injections of the same antigen formulated with SAF-1. Total ERA antibody and neutralizing antibody titers were determined periodically; however, during the course of immunization, both remained unchanged and decreased rapidly after the injections were discontinued (Figure 6A).

In HIV-infected persons, the majority of HIV-neutralizing antibodies are directed against the third hypervariable region of the external envelope glycoprotein, termed the V3 loop (Putney, S.D., Matthews, T.J., Robey, W.G., Lynn, D.L., Robert-Guroff, M., Mueller, W.T., Langlois, A.L., Ghrayeb, J., Petteway, S.R., Weinhold, K.J.,

Fischinger, P.J., Wong-Staal, F., Gallo, R.C. & Bolognesi, D.P. (1986) *Science* 234, 1392-1395; Rusche, J.R., Kavaherian, K., McDanal, C., Petro, J., Lynn, D.L., Grimaila, R., Langlois, A., Gallo, R.C., Arthur, L.O., Fischinger, P.J., Bolognesi, D.P., Putney, S.D. & Matthews, T.J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85, 3198-3202; and LaRosa, G.J., Davide, J.P., Weinhold, K., Waterbury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.J., Dressman, G.R., Boswell, R.N., Shadduck, P., Holley, L.H., Karplus, M., Bolognesi, D.P., Matthews, T.J., Emini, E.A. & Putney, S.D. (1990) *Science* 249 932-935). Antibodies to epitopes within the loop abrogate virus infectivity, probably by preventing fusion of the viral envelope to the target cell membrane. Neutralizing antibodies to V3 epitopes can, in fact, be added as long as 40 to 60 minutes after virus binds to the cell and still prevent infection (Nara, P.L., (1989) in *Vaccines 89*, eds. Lerner, R.A., Ginsberg, H., Chanock, R.M. & Brown, F. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) pp. 137-144). Therefore, to determine whether immunization with the V3 loop would boost neutralizing antibody titers, C-339 was injected with an oligopeptide of 25 amino acids, having the V3 sequence of HIV-$1_{BRU(IIIB)}$, cross-linked to KLH and formulated with SAF-1. No change in EIA titer was observed (Figure 6A), but a significant increase in neutralizing antibody titers, which were sustained for several months, was obtained following the second immunization at week 108 (Figure 7A).

Another chimpanzee, C-433, that had been primed by vaccination with VV-1139 (Kieny, M.P., Lathe R., Riviere, Y., Dott, K., Schmitt, D., Girard, M., Montagnier, L. & Lecocq, J.P. (1988) *Prot. Engineering* 2, 219-226), was immunized repeatedly with 125-250 μg each of recombinant soluble gp160*env*, p18*gag*, p27*nef* and p23*vif* (Table 1). The anti-HIV antibody response induced by this regimen was clearly transient, with titers rising sharply after each booster injection and then decreasing rapidly (Figure 6B). The neutralizing antibody and EIA titers of C-433 fluctuated in

parallel. Finally, C-433 was injected with the same V3 peptide-KLH conjugate as C-339, according to the same immunization protocol. Neutralizing antibody titers increased significantly after the second injection of the V3-peptide conjugate and remained high thereafter (Figure 7A); a third immunization 4 months later (week 126) elicited no change in titers.

At the time C-433 first received the purified recombinant proteins (48 weeks), a third chimpanzee, C-499, received an IM injection of purified gp160*env* and p18*gag* formulated with SAF-1. C-499 received six booster inoculations of the same antigens, followed by a series of four injections of a mixture of 21 free (unconjugated) V3 peptides (Myers, G. (1990) in *Human Retroviruses and AIDS*, eds. Myers, G., Josephs, S.F., Wong-Staal, F., Rabson, A.B., Smith, T.F. & Berzofsky, J.A. (Los Alamos National Laboratory, Los Alamos, NM) in SAF-1. As with C-339 and C-433, C-499's EIA titers declined rapidly after immunization with the purified HIV antigens, and there was no detectable effect of the V3 peptides on EIA titer. There was, however, a significant increase in neutralizing antibody titers (to > 2000) following the V3 peptide inoculations (Figure 7B).

Challenge with Infectious HIV. Because sustained neutralizing antibody titers were achieved, chimpanzees C-433, C-339 and C-499 were challenged by IV inoculation of 100 $TCID_{50}$ (40 chimpanzee infectious doses) of HIV-1. At the time of challenge, 50% neutralization titers by an immunofluorescence inhibition assay were 1:2000, 1:280-350 and 1:2000, and 90% neutralization titers by a syncytia-inhibition assay (Nara, P.L., Hatch, W.C., Dunlop, N.M., Robey, W.G., Arthur, L.O., Gonda, M.A. & Fischinger, P.J. (1987) *AIDS Res. Human Retroviruses* 3, 283-302) were 1:512-1024, 1:128 and 1:1024 for chimpanzees C-433, C-399 and C-499, respectively. Because immunization of C-499 was initiated at a different time from the other two animals, challenge of C-499 occurred 6 months after that of C-399 and C-433, but was done at the same time as that of a naive

control animal, C-087. Virus was isolated from C-087's PBMC at 2 weeks post-inoculation (PI) as well as at all subsequent times, showing that a 1:100 dilution of the HIV-1 stock readily infected chimpanzees under our conditions.

Attempts to Isolate HIV from Immunized and Challenged Chimpanzees. At various times after challenge with HIV-1, three methods were used to assess the infection status of the immunized animals. First, attempts to detect HIV sequences in lymphoid cells by PCR were made periodically (Laure, F., Rouzioux, C., Veber, F., Jacomet, C., Courgnaud, V., Blanche, S., Burgard, M., Griscelli, C. & Brechot, C. (1988) *Lancet* 2, 538-541; Mullis, K.B. & Faloona, F.A. (1987) *Methods Enzymol.* 155, 335-350; and Kwok, S. & Kellogg, D.E. (1990) in *PCR Protocols: A Guide to Methods and Applications*: eds. Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J. (Academic Press, Inc., San Diego, CA) pp. 337-347). DNA samples obtained from PBMC of the three chimpanzees at 3 weeks and 3 and 6 months after challenge were tested. Bands with the expected electrophoretic mobility were detected in DNA from control HIV-infected chimpanzee, but not in PBMC from the vaccinated and challenged animals or from a control naive animal (data not shown). At 6 months after challenge, nested sets of primers were used to perform PCR analysis on DNA from both PBMC and lymph node tissue of the challenged and control chimpanzees (Mullis, K.B. & Faloona, F.A. (1987) *Methods Enzymol.* 155, 335-350). This technique is more sensitive than standard PCR, and in these experiments (repeated at least seven times on all samples), approximately one molecule of viral DNA was found to produce a strong signal when present in 1.5×10^5 cell-equivalents of DNA. All PBMC and lymph node samples were consistently negative except those from a previously infected chimpanzee, which were always positive (Figure 8). Thus, at 6 months after challenge, viral DNA was not present in PBMC and lymph node tissues at a frequency greater than one copy per 10^6 cells.

Second, at weeks 2, 4, 6 and 8, and at monthly intervals thereafter, attempts were made to isolate virus from PBMC by

cocultivation of the chimpanzees' PBMC with lymphocytes obtained from normal humans (Fultz, P.N., McClure, H.M., Swenson, R.B., McGrath, C.R., Brodie, A., Getchell, J.P., Jensen, F.C., Anderson, D.C., Broderson, J.R. & Francis, D.P. (1986) *J. Virol.*, 58, 116-124). Because $CD8^+$ cells have been shown to suppress virus replication not only in HIV-infected humans (Walker, C.M., Moody, D.J., Stites, D.P. & Levy, J.A. (1986) *Science* 234, 1563-1566; and Tsubota, H., Lord, C.I., Watkins, D.I., Morimoto, C. & Letvin, N.L. (1989) *J. Exp. Med.* 169, 1421-1434) and chimpanzees (P.N.F., unpublished data), but also in SIV-infected macaques (Tsubota, H., Lord, C.I., Watkins, D.I., Morimoto, C. & Letvin, N.L. (1989) *J. Exp. Med.* 169, 1421-1434), in some experiments chimpanzee PBMC were depleted of $CD8^+$ lymphocytes before cultures were established. In contrast to virus recovery from the control animal, C-087, virus was not recovered from either total PBMC or $CD4^+$-enriched cells from C-339, C-433, or C-499 at any time during the first 6 months of follow-up. At 6 months PI, inguinal lymph node biopsies were performed on all animals as well as on uninfected and HIV-infected control chimpanzees. Upon cocultivation with normal human PBMC, virus was recovered from the lymph node of the infected control, but not from those of the immunized and challenged chimpanzees (data not shown). Despite the fact that all attempts to detect virus during the first 6 month after challenge had failed, virus was isolated from C-433 cocultivation of PBMC obtained at 32 weeks and thereafter of bone marrow obtained 37 weeks after challenge.

Lastly, the challenged animals were monitored for possible seroconversion to HIV antigens that were not included in their immunization regimens. Immunoblot analysis (Diagnostic Pasteur) showed that C-433 and C-499, which had been immunized with, among other antigens, p18*gag* but not p25*gag*, did not seroconvert to p25 during 7 months follow-up; however, at 32 weeks (7½ months) PI, a faint p25 band was observed on immunoblots for C-433, which increased in intensity with succeeding serum samples (Figure 9). For

C-339, which had been immunized with whole inactivated HIV, there were no detectable increases in EIA antibody titers or in apparent levels of antibodies to any HIV-specific proteins (Figure 9). Also, using purified antigens in immunoblot assays, no antibodies to the *vif* or *nef* proteins were detected in serum from C-339 during 12 months follow-up.

The results presented here, as well as those reported by Berman and colleagues (Berman, P.W., Gregory, T.J., Riddle, L., Nakamura, G.R., Champe, M.A., Porter, J.P., Wurm, F.M., Hershberg, R.D., Cobb, E.K. & Eichberg, J.W. (1990) *Nature (London)* 345, 622-625), clearly show that it is possible to elicit a protective immune response in chimpanzees with various HIV-1 antigens. It has been shown that C-499 was protected against establishment of HIV infection, at least through 7 months follow-up, that C-339 was protected for 1 year, and that C-433 was protected partially, as evidenced by the 7-month delay in appearance of virus. It is possible, however, that C-433 also might have been fully protected if the challenge dose had been the same as that used by others (Berman, P.W., Gregory, T.J., Riddle, L., Nakamura, G.R., Champe, M.A., Porter, J.P., Wurm, F.M., Hershberg, R.D., Cobb, E.K. & Eichberg, J.W. (1990) *Nature (London)* 345, 622-625), which was fourfold lower than the dose used herein. Protection was demonstrated by: (1) failure to recover virus from PBMC during monthly attempts and from lymph node tissue at 6 months PI: (ii) negative hybridization signals in PCR analysis of DNA from PBMC at various intervals and from lymph nodes at 6 months PI, and (iii) the absence of antibody responses that normally follow a primary HIV infection or that are characteristic of anamnestic responses in previously vaccinated and challenged animals (Berman, P.W., Groopman, J.E., Gregory, T., Clapham, P.R., Weiss, R.A., Ferriani, R. Riddle, L., Shimasaki, C., Lucas, C., Lasky, L.A. & Eichberg, J.W. (1988) *Proc. Natl. Acad. Sci. U.S.A.* 85 5200-5204; Arthur, L.O., Bess, J.W., Waters, D.J., Pyle, S.W., Kelliher, J.C., Nara, P.L., Krohn, K., Robey, W.G., Langlois, A.J., Gallo, R.C. & Fischinger, P.J. (1989) *J. Virol.* 63,

5046-5053; Girard, M., Kieny, M.P., Gluckman, J.C., Barre-Sinoussi, F., Montagnier, L. & Fultz, P. (1990) in *Vaccines for Sexually Transmitted Diseases* eds. Meheus, A. & Spier, R. (Butterworth Co., Ltd., London), pp. 227-237).

That C-433 appeared to be protected for 7 months, but actually was infected from time of challenge, despite repeatedly negative results for virus isolation and detection by PCR, is worrisome and underscores the fact that HIV can be sequestered such that it defies detection by both virologic and serologic criteria. A similar occurrence was reported (Desrosiers, R.C., Wyand, M.S., Kodama, T., Ringler, D.J., Arthur, L.O., Sehgal, P.K., Letvin, N.L., King, N.W. & Daniel, M.D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86 86, 6353-6357) for a macaque immunized with inactivated whole virus and then challenged with infectious SIV. In that study, virus was not recovered initially until 32 weeks and an anamnestic response was not observed until 39 weeks after challenge. The observation in natural HIV infections that persons remained seronegative by conventional tests for extended times, but HIV was detected by PCR or virus isolation (Ranki, A., Valle, S.L., Krohn, M., Antonen, J., Allain, J.P., Leuther, M., Franchini, G. & Krohn, K. (1987) *Lancet* 2, 589-593; and Jehuda-Cohen, T., Slade, B.A., Powell, J.D., Villinger, F., De, B., Folks, T.M., McClure, H.M., Sell, K.W. & Ahmed-Ansari, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 3972-3976), suggests that high-risk individuals, such as sexual partners of HIV-infected persons, possibly could be infected despite negative serologic, virologic or PCR analyses.

In view of the complex regimen of immunization undergone by the three chimpanzees, it is difficult to determine which of the many antigens and/or antigen formulations were instrumental in eliciting partial protection. C-339 was immunized successively with inactivated HIV, purified gp160, and the V3 peptide-KLH conjugate. C-433 was immunized first with a vaccinia virus-gp160*env* recombinant, then with a mixture of purified *env*, p18*gag*, *nef* and *vif* antigens, and

finally with the V3 peptide-KLH conjugate. The simplest immunization regimen was that of C-499; it consisted of purified gp160*env* and p18*gag* followed by unconjugated V3 peptides. The antigens that were common to the three animals were gp160*env*, p18*gag* and the V3 peptide, but their relative importance remains to be determined. Adequate protection might require multiple antigenic determinants found on more than one viral protein, and/or multiple presentations of the same antigenic determinant.

It is of interest that previously tested prototype vaccines (Berman, P.W., Groopman, J.E., Gregory, T., Clapham, P.R., Weiss, R.A., Ferriani, R. Riddle, L., Shimasaki, C., Lucas, C., Lasky, L.A. & Eichberg, J.W. (1988) *Proc. Natl. Acad. Sci.* U.S.A. 85 5200-5204; Arthur, L.O., Bess, J.W., Waters, D.J., Pyle, S.W., Kelliher, J.C., Nara, P.L., Krohn, K., Robey, W.G., Langlois, A.J., Gallo, R.C. & Fischinger, P.J. (1989) *J. Virol.* 63, 5046-5053; Girard, M., Kieny, M.P., Gluckman, J.C., Barre-Sinoussi, F., Montagnier, L. & Fultz, P. (1990) in *Vaccines for Sexually Transmitted Diseases* eds. Meheus, A. & Spier, R. (Butterworth Co., Ltd., London), pp. 227-237; and Hu, S.L., Fultz, P.N., McClure, H.M., Eichberg, J.W., Thomas, E.K., Zarling, J., Singhal, M.C., Kosowski, S.G., Swenson, R.B., Anderson, D., C. & Todaro, G. (1987) *Nature (London)* 328, 721-723) that did not elicit significant titers of neutralizing antibodies in chimpanzees were not effective in preventing experimental infection of the animals. The observation that sustained neutralizing antibody titers were reached in C-339 and C-433 after two injections of the V3 peptide-KLH conjugate and in C-499 after three injections of V3 peptides (Figure 7), suggests that V3 might be seen differently by the chimpanzee immune system when presented as a peptide than when presented as part of the gp160/120*env* molecule. We have found by immunoaffinity chromatography that virtually all HIV-neutralizing activity in the serum of the protected chimpanzees could be adsorbed by the V3 peptide (unpublished data of A.P.). The booster inoculations of the V3 peptide(s) might explain why

immunization with gp160 resulted in protection of chimpanzees in the subject experiments, but not in those reported by Berman et al. (Berman, P.W., Gregory, T.J., Riddle, L., Nakamura, G.R., Champe, M.A., Porter, J.P., Wurm, F.M., Hershberg, R.D., Cobb, E.K. & Eichberg, J.W. (1990) *Nature (London)* 345, 622-625). In this latter study, two chimpanzees were protected after immunization with gp120, and these animals had three- to four-fold higher titers to the principal neutralizing determinant (PND) found in the V3 loop than the two animals not protected from infection.

The question of whether the protection observed in the present experiment was due solely to neutralizing antibodies or whether other immune mechanisms were involved remains unanswered. At time of challenge, antibody-dependent cellular cytotoxic activity was detected in the serum of C-339, but not in that of the other two chimpanzees. HIV-specific proliferative responses to the soluble proteins p18*gag*, gp160*env*, and p27*nef* (Bahraoui, E., Yagello, M., Billaud, J.N., Sabatier, J.M., Guy, B., Muchmore, E., Girard, M. & Gluckman, J.C. (1990) *AIDS Res. Human Retroviruses* 6, 1087-1088; and Van Eendenburg, J.P., Yagello, M., Girard, M., Kieny, M.P., Lecocq, J.P., Muchmore, E., Fultz, P.N., Riviere, Y., Montagnier, L. & Gluckman, J.C. (1989) *AIDS Res. Human Retroviruses* 5, 41-50) were detected in PBMC from C-433 both before and after virus challenge, but not in PBMC from C-339. Interestingly, after immunization with the V3-KLH conjugate, C-433 displayed a sustained, strong T-helper cell reactivity to the V3 peptide, while C-339 had only a weak response. The responses of C-449 are currently under study. Repeated attempts to detect cytotoxic T lymphocytes (CTL) in PBMC of the vaccinated chimpanzees before, on the day of, and after challenge have failed. It appears, therefore, that the observed protection did not correlate with the T-helper cell or CTL activity.

The results presented here indicate that HIV vaccines can induce protection against virus infection. The high neutralizing antibody response induced by the V3 peptide was

type specific; serum from the vaccinated animals at time of challenge neutralized the more diverse HIV-1 isolates RF and MN only marginally (unpublished data). Therefore, it will be necessary to design a vaccine that will induce high titers of neutralizing antibodies to the many HIV variants, but the recent identification (LaRosa, G.J., Davide, J.P., Weinhold, K., Waterbury, J.A., Profy, A.T., Lewis, J.A., Langlois, A.J., Dressman, G.R., Boswell, R.N., Shadduck, P., Holley, L.H., Karplus, M., Bolognesi, D.P., Matthews, T.J., Emini, E.A. & Putney, S.D. (1990) *Science* 249 932-935) of PND sequences with which a majority of sera from HIV-infected persons react may make this less formidable than previously thought. The apparent success in protecting two chimpanzees and suppression of virus for an extended period in a third animal justify further efforts to develop an HIV vaccine, with the expectation that it will provide long-lasting protective immunity in humans.

* * *

Further studies were conducted to ascertain the validity of the dual immunization procedure (priming with gp160 followed by boosting with synthetic peptides with the sequence of the V3 loop of gp120); to compare 3 adjuvants: $Al(OH)_3$, the Syntex adjuvant, SAF-1, and incomplete Freund adjuvant (IFA); and to test an accelerated schedule of immunization: gp160 at 0 and 1 month, the V3 peptide at 3 and 4 months, and both gp160 and V3 as a last boost at 6 months.

The experiment was carried out in Rhesus macaques (4 animals per lot) using 100 μg of gp160 BRU for priming and a mixture of 200 μg each of V3-BRU (gp120 amino acid residues 302-335) and V3-MN (same residues) for boosting. The animals were bled at monthly intervals and anti-V3 and anti-gp antibody (Ab) titers were determined by ELISA. Neutralizing Ab titers were determined by the inhibition of immunofluorescent foci formation assay.

Anti-gp160 Ab were measured by ELISA using plaques coated with purified gp160 BRU. A fast anti-gp160 Ab response was observed in the 3 groups of animals (Fig. 10), but the response to the antigen in the groups with IFA and SAF-1 was from 5 to 10 fold higher than that in the group with alum. Injection of V3 peptides had no effect on anti-gp160 titers. Titers were boosted several fold upon recall injection of gp160 at 6 months, but again, the group with alum had a 2-8 fold lower response than the other 2.

Anti-V3 Ab were measured by ELISA using plaques coated with the BRU peptide. The response to V3 was clearly biphasic in all groups, with a strong booster effect seen upon injection of the V3 peptide at 3 months (Fig. 11). Thus, anti-V3 titers increased 10 fold between months 3 and 4 and then plateaued, confirming the remarkable booster effect of a V3 peptide injection in gp160-primed animals. This was observed irrespective of the adjuvant used in the experiment.

The initial response to V3, measured at month 3, was, however, 5-6 fold higher in the SAF-1 and IFA groups than in the group with alum. The final anti-V3 titers were altogether about 10 fold higher in the former 2 groups than in the latter. A two-step immunization schedule can be defined as follows:

priming: gp160 at 0 and 1 month
boosting: V3 peptides at 3 months
second boosting: gp160 + V3 peptides at 6 months.

The second boost can be placed at a later time, such as 12 months, to increase further the anamnestic response.

All pre-immune sera were negative for neutralizing Ab. Titers of neutralizing Ab measured at one month after the second boost (month 7) were the following:

	Adjuvant		
Monkeys	$Al(OH)_3$	SAF-1	IFA
1	60	140	> 450
2	neg	135	340
3	123	> 450	292
4	neg	> 450	440

Here again, there was a definite advantage in using SAF-1 or incomplete Freund adjuvant over using alum, although the relative difference in titers was somewhat less pronounced between the various groups.

In conclusion, a fast 2-step anti-HIV immunization schedule for primates is able to induce high anti-V3, high anti-gp160, and high neutralizing Ab responses. This schedule includes:

gp	gp	V3	V3	gp + V3
0	1	3	4	6

An alternative to that schedule could be:

gp	gp	V3	gp + V3
0	1	3	6

There is an advantage in using the Syntex adjuvant SAF-1 or incomplete Freund adjuvant rather than Alum [$Al(OH)_3$], as final Ab titers are from 5 to 15 fold higher with the former 2 adjuvants as compared to the latter.

* * *

Further studies on monkeys are reported in the following table.

<u>Neutralizing antibody titers</u> - Sera from the 5th and 7th month of immunization were monitored for HIV-1 neutralizing antibodies as described in Material and Methods.

Group	Monkey number	Neutralizing antibody titer		
		Syncitium inhibition		Immunofluorescent foci inhibition
		5 months	7 months	7 months
A	51	8	<2	60
	52	2	<2	<50
	68	16	4	120
	69	4	4	<50
B	53	256	256	>450
	57	256	256	340
	58	256	128	290
	59	512	128	440
C	48	16	16	140
	60	16	64	135
	61	128	128	>450
	64	64	64	>450

VACCINE PROTECTION OF CHIMPANZEES AGAINST CHALLENGE WITH HIV-1-INFECTED PERIPHERAL BLOOD MONONUCLEAR CELLS

Recent studies have demonstrated that, irrespective of stage of infection or disease, blood of persons infected with the human immunodeficiency virus (HIV) contains both virus-infected cells (also called cell-associated virus) and cell-free virus (). These findings imply that transmission of HIV may occur with either or both forms of virus. Although data regarding the quantity and primary form of HIV in vaginal and seminal fluids are limited (), it probably can be assumed that both cell-free and cell-associated virus are also transmitted through sexual contact. Therefore, any effective vaccine against HIV must protect against both forms of virus as well as from transmission via mucosal surfaces (sexual) and intravenously (through exchange of blood).

Animal model systems employing either HIV-1 infection of chimpanzees or infection of various macaque species with HIV-2 or the simian immunodeficiency virus (SIV) have been used to demonstrate that vaccination can elicit immune responses capable of protecting against infection with these viruses (). However, in all cases, protection was demonstrated only against challenge with relatively low doses of infectious cell-free virus. In the present study we determined (i) whether serum and/or peripheral blood mononuclear cells (PBMC) from HIV-immunized chimpanzees could prevent transmission of cell-associated HIV-1 *in vitro*, and (ii) whether chimpanzees previously immunized with various HIV-1 antigen preparations would be protected against intravenous challenge with PBMC from an HIV-infected chimpanzee.

As reported previously (), chimpanzee C-339 was immunized with various HIV-1 antigens and was subsequently challenged with an intravenous injection of 100 $TCID_{50}$ of cell-free HIV-1. This animal had remained virus negative by multiple criteria and did not develop an anamnestic antibody response to the virus through 40 weeks after challenge. Because unrelated *in vivo* studies had indicated that immune stimulation induced increases in HIV-1 expression in long-term infected chimpanzees (), and to insure that C-339 had

indeed been protected from infection, we attempted to reactivate or induce detectable expression of putative latent virus by stimulating the animal's immune system. At week 40 after challenge, C-339 was inoculated with the Syntex adjuvant formulation, SAF-1, and at weeks 44 and 48, the animal was injected with a mixture of HIV-1 antigens (inactivated HIV-1_{LAV-1}: recombinant antigens gp160*env*, p25- and p18-*gag*; and peptides representing the V3 immunodominant loop, all formulated with SAF-1). While none of these inoculations resulted in detection of virus by cocultivation of C-339's PBMC with normal human PBMC, the last two injections of HIV-1 antigen did serve as booster immunizations; increases in total anti-HIV-1 (Figure 1) and neutralizing (data not shown) antibody titers were observed.

To obtain an indication as to whether C-339's level of HIV-specific immunity might be sufficient to prevent infection by HIV-infected cells, *in vitro* assays for both humoral and cell-mediated inhibition of transmission were performed. We first tested whether serum from chimpanzee C-339 could prevent transmission of infectious virus from PBMC from an HIV-1-infected chimpanzee to PHA-stimulated normal human PBMC. As a positive control, serum (from an HIV-1-infected chimpanzee) that completely inhibited cell-to-cell transmission (P.N.F., manuscript in preparation) was included in each assay. Compared to serum obtained from C-339 prior to immunization, which had no inhibitory activity, serum from weeks 0 (at time of challenge with cell-free virus) and 52 inhibited virus transmission and production by 68% and 75%, respectively, whereas serum from week 24 inhibited virus production by only 33% (Figure 2A). The week 24 value is probably a reflection of gradual loss of inhibitory activity after the initial virus challenge, and that at week 52, of an increase in inhibitory activity due to the two HIV-1 booster injections given to C-339 at weeks 44 and 48.

Second, we tested whether PBMC from C-339, when used as indicator cells, would prevent transmission and replication

of virus when cocultivated with PBMC from an HIV-1-infected chimpanzee (C-087). PBMC from C-339 were added at a fixed concentration (2-3 x 106 cells/well) to wells of 12-well tissue culture plates. C-087's PBMC were serially diluted 1:4, and cells from each dilution were added to duplicate wells containing PBMC from C-339 (or normal human or chimpanzee PBMC, as controls), starting with a ratio of 1:1. Culture supernatants were monitored periodically for virus production by reverse transcriptase assay. Inhibitory activity was considered to be present in cells from the immunized animals if (i) a larger number of C-087's PBMC were required to yield virus-positive cultures within 6 weeks of observation, and (ii) there was a delay in time at which cultures became virus positive, compared with those cocultures established with PBMC from HIV-1-naive individuals. These assays indicated that C-339 had substantial inhibitory activity on week 40, which was before the two booster injections of HIV-1 antigens (Figure 2B). Although this inhibitory activity had declined by week 73, enrichment for CD8+ cells by magnetic bead depletion of CD4+ cells resulted in complete inhibition of virus recovery (Figure 2C). The apparent enhancement of infection with the CD4+-enriched population of C-339's PBMC probably is a function of the greatly increased number of cells capable of supporting replication of HIV-1.

Because the *in vitro* assays indicated that both serum and PBMC from C-339 had at least some ability to prevent cell-to-cell transmission of HIV-1, C-339 and a negative control chimpanzee, C-435, were challenged intravenously with HIV-1-infected PBMC. The challenge inoculum consisted of cryopreserved PBMC that were obtained from heparinized blood of a chimpanzee, C-087, that had been infected 14 weeks earlier with HIV-$1_{HTLV-IIIB}$ (as a positive control in another vaccine study []). A challenge inoculum consisting of PBMC from an HIV-1-infected chimpanzee was believed to most nearly approximate transmission that occurs between, for example, intravenous drug users. Since the minimal infectious dose of

HIV-infected cells required for infection of chimpanzees had not been determined, and because of the limited number of available chimpanzees, the dose of the challenge inoculum was selected empirically. This selection was based on the results of *in vitro* titrations of aliquots of the cryopreserved PBMC from chimpanzee C-087, using PHA-stimulated normal PBMC from both humans and chimpanzees as indicator cells (). From these assays, it was determined that there was an average of 382 infectious cells per 10^7 total PBMC in this cryopreserved stock. The two chimpanzees, C-339 and C-435, were inoculated intravenously with a volume of 1 ml, which contained 5.8×10^5 PBMC or 22 infectious PBMC. This number is a minimum estimate and is based on the assumption that one infected cell is sufficient for a culture to become virus positive.

Following inoculation, the animals were observed daily, and blood samples were obtained every 2 weeks for 8 weeks and at monthly intervals thereafter. Virus isolation attempts were performed by cocultivation of PBMC from each animal with PHA-stimulated normal human PBMC in 25-cm^2 tissue culture flasks. We also attempted to isolate virus from bone marrow biopsy samples obtained at 3 and 9 months and from lymph node biopsies at 6 and 11 1/2 months after inoculation of infected PBMC. At 4 weeks after challenge and at every time thereafter, virus was isolated from PBMC, as well as bone marrow and lymph node samples, from the control animal, C-435. In contrast, virus was not isolated at any time from PBMC, nor from bone marrow or lymph node biopsies, from the immunized chimpanzee, C-339. HIV-specific antibodies were detected in serum from C-435 initially at 8 weeks after challenge, and titers continued to rise through week 24 (Figure 1). However, no anamnestic response was detected in serum from C-339, and antibody titers to HIV-1 diminished slightly, then remained stable.

These results, therefore, indicated that it was possible to prevent transmission of infection by HIV-infected cells by prior immunization. As confirmation, two additional

immunized chimpanzees were challenged with an equivalent number of infectious cells using an aliquot of the same cryopreserved PBMC from chimpanzee C-087 (Table 1). One of these chimpanzees, C-499, like C-339, had been immunized and challenged previously with cell-free HIV-1 and had remained virus negative for 1 year (). The second chimpanzee, C-447, had been immunized initially with purified recombinant gp160*env*, p18*gag*, *vif*, and *nef* proteins in SAF-1, and then received booster immunizations with purified gp160*env* and p18*gag*, followed by peptides representing the principal neutralizing determinant (V3 loop) of HIV-$1_{HTLV-IIIB}$ and purified *nef* protein in SAF-1. Chimpanzee C-447 had not been exposed previously to infectious HIV-1 in any form.

Following challenge, with the same dose of approximately 22 infectious PBMC, these latter two chimpanzees were monitored biweekly, then monthly, for changes in HIV-specific antibody titers and for presence of virus in PBMC, bone marrow and lymph node. Antibody titers to HIV-1 in both animals remained stable, and virus was not isolated from any of the blood or tissue samples. At 7 months after challenge, C-499 was sacrificed due to congestive heart failure. Fragments of eight different tissues (including brain, spleen, various lymph nodes, kidney, liver and salivary gland) were minced with scissors; these tissue fragments, as well as PBMC and bone marrow, were then cocultivated with PHA-stimulated normal human PBMC. All cultures were virus negative throughout 6 weeks in culture, as monitored by reverse transcriptase assay. All PBMC, bone marrow and lymph node samples from the second animal, C-447, have been negative for virus on all attempts through 9 months of follow-up. Thus, three of three immunized chimpanzees were apparently protected from infection by HIV-1-infected cells. Since peripheral blood cells contain monocyte/macrophages as well as lymphocytes, the infected cell population was probably heterogeneous not only with respect to cell type but also according to levels of virus expression by individual cells. Although the inocula was prepared as PBMC suspended

in 1 ml of medium, it is highly likely that some of C-087's PBMC were actively producing HIV. It is possible, therefore, that the chimpanzee inocula actually consisted of a mixture of both cell-free and cell-associated HIV-1. These considerations further enhance the importance of our results.

At time of challenge with HIV-infected PBMC, C-447 and C-499 had fourfold lower HIV-1 EIA antibody titers (1:6400 versus 1:25,600), but four- to eight-fold higher neutralizing antibody titers (1:256 and 1:512 versus 1:64), compared with those of C-339. To assess further the potential of the *in vitro* serum and PBMC inhibition assays to predict possible vaccine-induced protection against cell-associated virus challenge, serum samples from C-447 and C-499 were tested. Serum obtained from C-447 and C-499 on day of challenge inhibited cell-to-cell transmission of HIV-1 by 25% and 52%, respectively. Because these levels of inhibition were less than the 75% inhibition of cell-to-cell transmission observed with serum from C-339 on the day it was challenged, this assay may not be a reliable predictor of protection against cell-associated challenge. PBMC from these two chimpanzees on the day of cell-associated challenge were tested in parallel with PBMC from C-339 (see Figure 2B, week 75). Results were equivalent to those obtained with C-339's PBMC from week 75; that is, PBMC from both animals exhibited no apparent inhibitory activity against transmission of virus from C-087's infected cells.

When C-339 had been protected from cell-associated HIV-1 challenge for 1 year (week 104 relative to the initial cell-free virus challenge of C-339), we again challenged this animal with an inoculum of cell-free HIV-$1_{HTLV-IIIB}$ that was equivalent to that used for the first challenge experiment 2 years earlier. Using another cryopreserved aliquot of the same virus stock (obtained from Larry Arthur, NCI-FCRF), 100 $TCID_{50}$ were injected intravenously in a total volume of 1 ml. HIV-1 was initially detected in PBMC from C-339 (by cocultivation with normal human PBMC) that were obtained 4 weeks after this third HIV-1 challenge, and an increase in

HIV-1 EIA antibody titer was observed at 6 weeks after challenge (Figure 1, week 110). Because C-339 had not received a booster immunization or been exposed to HIV-1 for 1 year prior to this second challenge with cell-free HIV-1, the immune response elicited by vaccination did not persist at a level sufficient to protect against this last exposure to virus. C-339 became infected despite the presence of a stable HIV-1 immune response, and infection was detected relatively soon after the third exposure to virus. This finding shows that C-339 was not inherently resistant to HIV-1 infection, and furthermore, underscores the significance of the observed protection against cell-associated HIV-1 challenge. The other surviving chimpanzee, C-447, will be challenged similarly when it has remained virus negative for 1 year.

The mechanism of protection of the three chimpanzees against challenge with HIV-infected cells is not known, but it is likely to be due to a combination of both humoral and cell-mediated immunity. In the *in vitro* assays with PBMC obtained on the days of challenge, only cells from C-339, but not from C-499 and C-447, exhibited significant inhibitory activity against recovery of HIV-1 from C-087's PBMC. This may have resulted from the fact that C-339 was boosted with multiple HIV-1 antigens 4 and 8 weeks prior to cell challenge, whereas C-499 had not been exposed to HIV-1 antigens for more than 1 year. Also, C-447 had received three booster immunizations with only V3 peptides and Nef protein during an interval 2 to 5 months earlier; these inoculations had resulted in more than a tenfold increase in neutralizing antibody titers, but no detectable increase in HIV-specific EIA antibody titers. That PBMC from C-339 subsequently lost the ability to prevent cell-to-cell transmission *in vitro* supports this possibility. Irrespective of this, it appears that neither of the *in vitro* assays, as performed with serum or PBMC, are predictive of protective immunity.

Because C-087 and the three chimpanzees that were challenged with HIV-infected PBMC from C-087 were not siblings, the possibility that the four animals shared identical major histocompatibility complex (MHC) haplotypes is extremely low. Thus, one would assume a priori that initial protection against C-087's PBMC, some of which had HIV antigens on their surface, was not mediated by classical MHC-restricted cytotoxic T-cell activity, even if present. To date we have been unable to detect CTL activity directly in peripheral blood lymphocytes from immunized chimpanzees (). The most likely cell-mediated mechanism of protection would appear to be antibody-dependent cellular cytotoxicity (ADCC), an activity previously detected in serum from C-339 (). As indicated above, it is likely that both HIV-specific antibodies and cell-mediated activities synergized to effect protection.

Ideally, a vaccine against any pathogen should be one that elicits long-lasting immunity following a minimal number of immunizations. While we have observed long-lasting, stable EIA and neutralizing antibody titers in our immunized chimpanzees, these were achieved with a large number of immunizations (no fewer than ?12?) over a minimum of 2 years. These regimens, to say the least, are not practical for use in Western nations, much less in developing countries. Based on studies to date in nonhuman primate models, it appears as though immunization against HIV-1 will require at least three inoculations initially and booster inoculations at unspecified intervals. If multiple inoculations are required, then they must be easily administered (such as orally), and the vaccine preparation must be stable under normal storage conditions. These latter two conditions are especially important relative to HIV-1 vaccine delivery to developing nations. Thus, although progress has been made to demonstrate that it is possible to elicit protection against

intravenous infection with both cell-free and cell-associated HIV-1, major problems remain to be resolved.

REFERENCES AND NOTES

Table 1. Immunization history of chimpanzees prior to challenge with HIV-infected cells.

	Chimpanzees			
	C-339	C-435	C-499	C-447
Immunization:	inactivated HIV	none	gp160,p18	gp160,p18
	gp160, V3-KLH		21 V3 pept	vif, nef, V3
Prior challenge with cell-free HIV-1:	yes	no	yes	no
Status at time of cell-associated challenge:				
EIA anti-HIV titer:	25,600	< 100	6,400	6,400
Neutralizing titer:	1:64	< 4	1:512	1:256
Virus recovery:	none	wk 4	none	none

Serum Neutralization of Cell-to-Cell Transmission

Chimpanzee	Serum date	% Inhibition
C-339	10/87	0
Roberta	3/88	98
C-339	8/89	68
	1/90	34
	8/90	75
C-499	1/90	18
	7/90	49
	1/91	52
C-447	1/89	12
	10/90	27
	1/91	25
C-433	8/89	35

6×10^5 PBMC from C-527, 4 mos. p.i.

Average of 3 experiments

The influence of adjuvants on the neutralizing antibody response of rhesus macaques to HIV-1 gp160 and env peptide.

INTRODUCTION

The envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-1) is made of two moieties that arise by proteolytic cleavage of a large precursor, gp160. The exterior surface glycoprotein gp120 corresponds to the amino-terminal region of gp160, whereas the transmembrane glycoprotein gp41 is derived from its carboxyl-terminal region (1, 2). The principal neutralization determinant (PND) for the virus has been mapped to the third hyper-variable domain (V3) of gp120, a conserved cysteine loop located at residues 303-338 for strain IIIB (3-5) (numbering according to ref 6). In addition, both gp120 and gp41 carry minor neutralization epitopes (for a review see ref 7). The neutralization epitopes of the V3 loop are of a sequential nature (8, 9) but the 3-D conformation of the loop seems to be important for reactivity (10, 11). PNDs from different HIV-1 isolates exhibit extensive sequence divergence (4, 12, 13), which explains why antibodies to the PND neutralize virus infectivity in a type specific manner. These antibodies also inhibit syncitia formation and virus spread from cell-to-cell. PND-targeted antibodies act at the level of fusion between the virus envelope and the membrane of target cells, or between the membranes of infected and non-infected target cells (4, 14).

Experiments in the HIV-1 chimpanzee model have shown that neutralizing antibodies play a major role in protection against experimental HIV infection (15, 16). Most of those animals that were not protected against challenge infection had no neutralizing antibodies at the time of challenge or very low levels of neutralizing antibody only (17). Passive protection of chimpanzees against virus challenge could be achieved through administration of an anti-V3 domain specific virus-neutralizing monoclonal antibody (Emini, personal communication). Similarly, passive protection of cynomolgus monkeys against HIV-2 or SIV could be achieved by

administration of high doses of neutralizing anti-HIV or anti-SIV serum, respectively (18). The induction of high titers of neutralizing antibodies is therefore an apparent requisite for the efficacy of HIV vaccines, perhaps because these antibodies are required for the establishment and maintenance of an anti-HIV-1 sterilizing immunity (19).

We previously observed that chimpanzees that had been primed by hyperimmunization with a variety of HIV-1 antigens, among which gp160, then boosted with either free or KLH-coupled PND peptides, developed high titers of PND-specific neutralizing antibodies and were protected against subsequent HIV challenge (16). The simplest immunization regimen able to induce protective immunity consisted of gp160<u>env</u> and p18<u>gag</u> followed by unconjugated PND peptides. There is little reason to believe that p18<u>gag</u> could be involved in protection. Thus, the minimal protective immunization regimen should consist of gp160 followed by boosting with the corresponding PND peptide. The efficacy of such a priming-booster immunization schedule is likely to depend on multiple parameters such as dose and physical status of the gp160 antigen, amount and sequence of the PND peptides, number and spacing of the injections, and also on the nature of the adjuvant. In this study, the efficacy of alum, incomplete Freund adjuvant (IFA), and Syntex adjuvant formulation 1 (SAF-1) (20) were compared in a simplified priming-booster immunization regimen in rhesus monkeys.

As will be shown, the efficacy of IFA and SAF-1 appeared to be comparable, whereas alum was a much less potent adjuvant. These results could have important bearing on the design of future HIV vaccines.

<u>MATERIALS AND METHODS</u>

<u>Antigens</u>

Construction of VV1163, the recombinant vaccinia virus used for the production of gp160 from the LAV-1 (LAI) isolate of HIV-1 (21) has been described previously (22). The gp160 gene carried by VV1163 was mutagenized at the gp120-gp41 cleavage site and deleted of the transmembrane domain. The

antigen was purified from the cell culture medium of VV1163-infected BHK-21 cells as described (22-24). PND peptide (LAI) was prepared by solid phase synthesis as a 34 amino acid residues peptide with the sequence C-RPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQA (residues 303-336 from the BH10 isolate) and resuspended in phosphate buffered saline (Neosystem, Strasbourg).

Immunization of monkeys

Monkeys were immunized by the I.M. route with 2 injections of 100 μg recombinant gp160 at 1 month interval, followed by 2 injections of 200 μg PND peptide at 3 and 4 months (see Fig. 1). All antigens were in final volume of 1 ml. Four monkeys (group A) were immunized with the antigens adsorbed to 0.2% aluminium hydroxyde (Superfos), another four animals (group B) with the antigens emulsified in 1 ml IFA (Difco) and the last four (group C) with the antigens emulsified in 1 ml SAF-1 (20) containing 1 mg threonyl-MDP per dose. All animals were boosted at 6 months by I.M. injections of both 100 μg gp160 and 100 μg PND peptide in the same respective adjuvants. The animals were bled at regular intervals (10-15 ml). Serum was stored frozen until subjected to the experiments described below.

HIV serology

Anti-PND and anti-gp160 antibody titers were determined by ELISA using microwell plaques (Nunc) coated with 0.10 μg PND peptide or 0.15 μg gp160 per well, respectively. Incubation with the appropriate dilutions of serum was for 1.5 hr at 37°C, after which sera were replaced by horseradish peroxydase-labeled rabbit anti-monkey immunoglobulin (Nordic) and incubation was continued for another 1.5 hr at 37°C. Bound enzyme activity was measured using orthophenylenediamine (Merck) with 0.03 % hydrogen-peroxide as a substrate. The reaction was stopped after 30 min. with sulfuric acid and absorbance was read at 490 nm in an automatic plate reader (Vmax ; Molecular Device Corporation). End point titers were calculated from a linearized standard curve obtained with a selected pool of positive macaque sera

used as an internal standard. Titers obtained correspond approximately to the reciprocal of the highest serum dilution that resulted in an optical density of at least 0.1.

To measure apparent affinity constant (K_A), serial dilutions of the LAI PND peptide were made on peptide coated plates and a dilution of each macaque serum that yielded an absorbance (Ao) between 1 and 1.5 in the titration EIA described above was added. EIA was then performed as already described. Dissociation constants (K_D) were calculated as described by Friguet et al (25). K_A (affinity constant) was calculated as $K_A=1/K_D$.

Neutralizing antibodies were measured by inhibition of immunofluorescent foci formation on H9-cells or inhibition of synicita formation on CEM-SS cells (26) using a IIIB virus stock. Except where otherwise stated, neutralization titers were defined as the reciprocal of the serum dilution that reduced foci formation by 50% or synicita formation by 90% as compared with control.

RESULTS

The strategy that was devised and followed for the immunization of rhesus macaques is outlined in Fig. 1. Briefly, 3 groups of 4 monkeys were immunized with the same antigen preparations mixed with either alum (group A), IFA (group B) or SAF-1 (group C). The animals were primed with two injections of recombination HIV-1 gp160 given one month apart, then boosted with two injections of PND peptide on the 3rd and 4th months, followed by a last booster injection of both gp160 and PND peptide at 6 months. Antibody responses to whole gp160 and to the PND of the same HIV-1 isolate (LAI) as used for immunization, as well as neutralizing antibody responses to HIV-1 IIIB were monitored regularly during the 6 month immunization period and an additional 6 month follow-up.

Anti-gp160 antibody titers induced by the two initial injections of gp160 were approximately ten times higher in the IFA and SAF-1 groups than in the alum group (B, C, and A, respectively, table 1 and Fig. 2). These titers increased

only slightly in response to the injections of PND peptide, and began declining thereafter. The six month booster injection with gp160 plus peptide led to a 4-10 fold anamnestic antibody response to all three groups, but, again, the gp160 antibody titer in the alum group remained about one order of magnitude lower than the IFA or SAF-1 groups (Fig. 2A). After the peak at 7 months, titers progressively declined but were still significantly elevated at the 12th month bleed, except in the alum group (table 1).

Anti-PND antibody titers remained low after one injection of gp160 and became moderately high after two injections (table 2). They were markedly (3 to more than 20 fold) enhanced by the first injection of PND peptide, confirming our previous observation in chimpanzees (16). However, no further increase in titer was observed after the second injection of PND peptide (Fig. 2B). In several animals, that injection was actually followed by a drop in anti-PND titer (table 2). Similarly, the effect of the booster injection at 6 months on the anti-PND titer was of very limited amplitude, after which titers steadily declined. Anti-PND titers at 7 months were thus paradoxally lower than those at 4 months. The difference was significant at the 1% level. This suggests that too many injections of PND peptide could actually lead to some sort of immune paralysis. Anti-PND titers in the alum group remained at all times about one order of magnitude lower than those in the IFA and SAF-1 group and reached low level values at 12 months.

Neutralizing antibody titers were determined at 5 months (one month after the second injection of PND peptide) and again at 7 months (one month after the 6 month booster). As shown in table 3, the highest neutralizing titers were generated in the IFA group (B) and the SAF-1 group (C). Neutralizing titers in the alum group (A) remained definitely lower and some animals even scored negative in that group when measured at 7 months by syncitium inhibition assay. Most neutralizing antibody titers were as high or even higher immediately after the 2 injections of PND peptide (at 5

months) than after the combined gp160 and peptide booster injection (at 7 months), again suggesting that the repetition of injections of PND peptide might induce a certain degree of immune paralysis.

The full time-course of the neutralizing antibody response was followed on two animals of group B and two animals of group C (Fig. 3). Neutralizing titers induced by the two injections of gp160 were boosted about 50 fold following the injections of PND peptide. This is in agreement with our previous observation that high HIV neutralizing antibody titers could be induced in chimpanzees through priming with gp160 followed by boosting with PND peptides (16). The subsequent decay of the neutralizing antibody response was at the rate of approximately 50 % every 6 weeks (Fig. 3).

To study the possible correlation between the anti-PND antibody response and the neutralizing antibody response in the immunized animals, neutralizing titers determined by the syncitium inhibition assay were plotted as a function of anti-PND peptide titer (Fig. 4). A clear correlation between both titers was observed for all the animals. For the 5 month time point, the correlation coefficient computed for the 12 animals together was $r = 0.85$ ($P < 0.001$), and for the 7 month time point, $r = 0.89$ ($P < 0.001$). The fact that correlations were highly significant in spite of the limited number of animals leads to suggest that the relationship between both parameters was very strong.

Finally, apparent affinity constants in solution of the different monkey sera for the PND peptide was determined by ELISA (25). No major difference was observed between the 3 groups of immunized animals (data not shown).

DISCUSSION

The aim of the present study was to compare the efficacy of three different adjuvant formulations with respect to their capacity to induce HIV-1 neutralizing antibodies in rhesus macaques in a gp160 priming - PND peptide booster immunization schedule limited to 5 injections over the course

of a 6 month immunization period. The data presented here show that high anti-gp160, anti-PND, and neutralizing antibody titers could be raised in response to such a short regimen of immunization, provided the adjuvant was suitably chosen. In agreement with reports from others (27-31) aluminium hydroxyde was unable to provide help for a strong anti-HIV-1 antibody response. By contrast, IFA and the threonyl-MDP base SAF-1 were both found suitable to induce high-titered anti-HIV antibody responses. A similar low grading of alum in comparison to SAF-1 and IFA was observed by Hart et al (32) when studying the response of rhesus macaques to T1-SP10, a synthetic peptide made of the HIV-1 PND peptide and the envT1 Th-cell epitope from gp120. Alum was also the poorest of the three adjuvants with respect to the induction of antibodies to the HIV-1 PND in rabbits (33). In our study, the highest HIV-neutralizing titers measured by either the immunofluorescent foci or the syncitium inhibition assays were observed in monkeys receiving IFA. Neutralization titers in monkeys receiving SAF-1 were slightly lower, but still highly significant. Titers in the animals receiving the aluminium hydroxide-adjuvanted antigens were disappointingly low. In all three groups of animals, the peak of neutralizing antibody titer was reached after the injection of PND peptide, confirming the advantage of the dual gp160-PND peptide immunization regimen previously described (16, 19). It should be emphasized that there was no significant difference in the mean apparent half-life of the gp160- and PND-specific antibodies nor in the apparent affinity constants of the PND-specific antibodies between the 3 groups of immunized animals. The difference between alum and the other two adjuvants was, therefore, only in the actual level of antibodies elicited.

We have observed in previous experiments that immunized chimpanzees with HIV-1 neutralizing antibody titers of greater than 1:32 at time of challenge were protected against intravenous virus challenge (16, and unpublished observations). Taking this tier as an indicative threshold

level, it is interesting to note that 3 of 4 rhesus monkeys in the SAF-1 group and 4 of 4 in the IFA group, but none of the 4 animals in the alum group, had titers above that level as early as at the 4th month of immunization. Altogether, the 6 months immunization schedule followed in this study was able to elicit high neutralizing antibody titers in 7 of 8 animals in pooled groups B and C. We suggest that in future experiments, the injection of PND peptide at 4 months ought to be omitted, in view of its possible induction of immune paralysis, thus reducing the total number of injections to 4 only. The possibility of a further decrease of the number of injections and/or of the dose of gp160 and PND peptides used for immunization awaits further experiments.

Immunization of chimpanzees against HIV-1 using aluminium hydroxide as an adjuvant has usually yielded disappointing results. Thus, titers of neutralizing antibodies elicited in chimpanzees by alum-adjuvanted gp120 have most often remained low (27, 29, 31), which probably explains why no protection was observed upon subsequent virus challenge of the animals (28, 29). We recently immunized three chimpanzees with gp160 in alum, then boosted them with PND peptides, also in alum, but in view of the low neutralizing antibody titer induced by this immunization regimen, none of the animals was challenged (unpublished observation). By contrast, studies by Berman et al (15) have shown that chimpanzees immunized with gp120 in alum, but not with gp160, developed HIV-1 neutralizing antibodies and were protected against challenge with a moderate dose of cell-free virus. Whether the difference between this observation and the former ones has to do with the nature, the physical state (fully native versus perhaps partly denatured) and/or the dose of the immunogen remains to be determined.

The reason why alum was not able to elicit as strong an immune response as IFA or SAF-1 in this and other studies may have to do with the fact that aluminium hydroxide is a mineral carrier whereas both IFA and SAF-1 contain oil. The importance of lipids for antigen presentation has been

previously recognized (20, 31, 34 35). Presentation of the HIV-1 PND to the immune system could understandably be made very different by the presence or absence of oil. The use of IFA in humans has already been described, with little adverse reactions reported (34, 36). IFA could therefore represent a useful adjuvant for future HIV vaccines in humans.

Remarkably, the 12 animals used in this study showed a strong, significant correlation between anti-PND and neutralizing antibody titers at the 5 months and 7 months time points, suggesting that the majority of the neutralizing antibodies were targeted to the PND, independent of the adjuvant used in the vaccine (Fig. 4). A similar correlation was observed in chimpanzees, although not as strict as observed here (unpublished observations).

These results have strong implications for HIV-1 vaccines. First, they show that adjuvants such as IFA or SAF-1 should be preferred to alum in view of their greater potency. Second, it has recently been shown that, in addition to the PND, gp120 also contains conserved, conformational neutralization epitopes that elicit broadly cross-reactive neutralizing antibodies (37-41). Antibodies to these conformational epitopes seem to neutralize virus infectivity by interfering with the binding of the virus to its CD4 receptor (38, 39, 42). The fact that the great majority of the neutralizing antibodies elicited by gp120 alone (15) or gp160 followed by PND peptides (16, and this study) is essentially directed to the PND alone, suggests that the conserved, conformational neutralization epitopes of gp120 are not visible to the immune system of primates under these conditions. It would be of major importance for the future development of HIV vaccines to find a way to improve or modify the mode of the delivery of the antigen(s) and/or to manipulate the immune response of the host so as to elicit such a broadly-neutralizing antibody response, short of which HIV-1 vaccines will run the risk of remaining isolate-specific and unable to cope with the wide antigenic variation of the virus in the field (6).

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Antibody titer x 10^{-3} at month

Group	Monkey number	1	3	4	5	6	7	
A	51	53.6	37.6	29.1	58.0	49.2	151.5	21.0
	52	18.9	34.2	25.6	21.0	4.6	39.3	3.4
	68	22.9	20.4	84.6	130	51.7	290.5	34.2
	69	14.8	33.8	201.5	56	40.0	302.5	23.2
B	53	38.2	452.2	2871.7	1200	562	2794.0	458.0
	57	71.1	578.3	4524.1	2000	702	4675.0	370.0
	58	138.5	705.4	1405.5	1365.1	292	1360.0	165.0
	59	56.5	505.3	2920.3	2100.0	374.0	1254.0	107.0
C	48	13.6	127.4	450.1	512.0	140.0	618.4	164.0
	60	97.5	279.4	150.1	180.9	109.0	1502.0	112.0
	61	360.4	494.4	1101.8	550.0	324.5	2080.0	322.0
	64	15.9	187.7	351.2	580.0	201.5	2350.0	220.0

Table 1 - Anti-gp160 ELISA titers Macaques injected as described in Fig. 1 using $Al(OH)_3$ (A), IFA (B) or SAF-1 (C) as an adjuvant, were bled at the indicated times and monitored for anti-gp160 antibodies by ELISA (see Material and Methods). All pre-immune sera were negative (< 10).

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Claims

1. A method of enhancing the immunogenicity of an envelope glycoprotein of a virus in a host, wherein the method comprises administering to the host at least one envelope glycoprotein of the virus and at least one peptide derived from the amino acid sequence of the envelope glycoprotein, and wherein the peptide comprises at least one virus-neutralization epitope, and wherein the envelope glycoprotein and the peptide are administered in an amount sufficient to induce neutralizing antibodies in the host.

2. Method as claimed in claim 1, wherein the virus is selected from the group consisting of HIV, SIV, HTLV-1, HTLV-2, FIV, and FeLV.

3. A method of enhancing the immunogenicity of an envelope glycoprotein of a virus, wherein the method comprises administering to a host at least one envelope glycoprotein of virus in an amount sufficient for priming vaccination and at least one peptide derived from the amino acid sequence of said envelope glycoprotein, wherein the peptide comprises at least one virus-neutralization epitope of said glycoprotein of virus, and the peptide is administered to the host in an amount sufficient to enhance the induction of neutralizing antibodies in the host to confer to the host long-lasting immunity against the virus.

4. Method as claimed in claim 3, wherein said envelope glycoprotein and said peptide are simultaneously administered to said host.

5. Method as claimed in claim 3, wherein said envelope glycoprotein is administered to said host, and then said peptide is administered to said host.

6. Method as claimed in claim 3, wherein said envelope glycoprotein is gp120 or gp160 of HIV.

7. Method as claimed in claim 3, wherein said at least one peptide comprises a mixture of said peptides, which are administered to the host in a free state not coupled to a carrier molecule.

8. Method as claimed in claim 7, wherein the peptides are coupled to a carrier molecule.

9. Method as claimed in claim 7, wherein the carrier is a lipopeptide.

10. Method as claimed in claim 3, wherein the envelope glycoprotein comprises a mixture of HIV-1 and HIV-2 envelope glycoproteins, and said peptide comprises a mixture containing at least one peptide having an HIV-1 neutralization epitope and at least one peptide having an HIV-2 neutralization epitope.

11. Method as claimed in claim 3, wherein the envelope glycoprotein is orally administered to the host.

12. Method as claimed in claim 3, wherein the envelope glycoprotein is parenterally administered to the host.

13. Method as claimed in claim 3, wherein the peptide is orally administered to the host.

14. Method as claimed in claim 3, wherein the peptide is intradermally administered to the host.

15. Method as claimed in claim 3, wherein said envelope glycoprotein is a mixture of gp160 glycoproteins from different HIV isolates (serotypes) and said at least one peptide is a mixture of the corresponding neutralization epitopes.

16. Method as claimed in claim 3, wherein said envelope glycoprotein is a mixture of glycoproteins gp120 from different HIV isolates (serotypes) and said at least one peptide is a mixture of the corresponding neutralization epitopes.

17. Method as claimed in claim 3, wherein said envelope glycoprotein is gp120 of HIV.

18. Method as claimed in claim 3, wherein the envelope glycoprotein and the peptide are administered in combination with an adjuvant to the host.

19. Method as claimed in claim 18, wherein the adjuvant is muramyl dipeptide in a lipid medium or incomplete Freund's adjuvant.

20. Method as claimed in claim 19, wherein the peptide is selected from the group consisting of *env*, *pol*, *gag*, *nef*, *vif*, antigen, and mixtures of said antigens.

21. Method as claimed in claim 19, wherein said mixture of said antigens comprises p27*nef* and p23*vif*.

22. Method as claimed in claim 19, wherein the peptide is at least one peptide selected from the group consisting of:

```
C-TRPNNNTRKR IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKK IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRGS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IYI--GPGRA FHTTGRIIGD -IRKAH-C
C-TRPYNNVRRS LSI--GPGRA FRTRE-IIGI -IRQAH-C
C-TRPGNNTRRG IHF--GPGQA LYTTGIV-GD -IRRAY-C
C-ARPYQNTRQR TPI--GLGQS LYTTRSR-SI -IGQAH-C
C-TRPNNNTRKS ITK--GPGRV IYATGQIIGD -IRKAH-C
C-TRPNNNTRKR ITM--GPGRV YYTTGQIIGD -IRRAH-C
C-TRPGSDKRQS TPI--GLGQA LYTTRGRTKI -IGQAH-C
C-TRPGSDKKIR QSIRIGPGKV FYAKGG---I -TGQAH-C
C-TRPNNNTKKG IAI--GPGRT LYAREKIIGD -IRQAH-C
C-TRPNNHTRKR VTL--GPGRV WYTTGEILGN -IRQAH-C
C-TRPGNNTRRG SHF--GPGQA LYTTGIVGDI -RRAY-C
C-TRPDNKITSRQ-TPI-GLGQA LYTTRIKGDI -RQAY-C
C-TRPNNNVRRR-HIHI-GPGRA FYTGEIRNI  -RQAH-C
C-TRPYKNTRQS-TPI--GLGQA LYTTRTKSI  -GQAH-C
C-TRPNNNTTRS-IHI--GPGRA FYATGDIIGTIRQAH-C
C-TRPNYNKRKR-IHI--GPGRA FYTTKNIIGDIRQAH-C
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23. Method as claimed in claim 19, wherein the peptide comprises the following amino acid sequence:

YNTRKSIRIQRGPGRAFVTIGKIGN.

24. Method as claimed in claim 19, wherein said at least one peptide is comprised of the major neutralization epitope (loop V3) of at least one HIV-1 isolate.

25. Method as claimed in claim 19, wherein said envelope glycoprotein is administered to said host, said at least one peptide is administered to said host after said

envelope glycoprotein, and thereafter a mixture comprising at least one envelope glycoprotein of said virus and at least one peptide derived from the amino acid sequence of said envelope glycoprotein is administered to said host.

26. Method as claimed in claim 25, wherein said envelope glycoprotein is gp160 of HIV.

27. Method as claimed in claim 26, wherein said at least one peptide is comprised of the major neutralization epitope (loop V3) of at least one HIV-1 isolate.

28. A composition for vaccinating a host against infection by a virus, wherein the composition comprises

(A) at least one envelope glycoprotein of the virus in an amount sufficient for priming vaccination in a host to which the envelope glycoprotein is administered; and

(B) at least one peptide derived from the amino acid sequence of said envelope glycoprotein, wherein the peptide comprises at least one virus-neutralization epitope of said glycoprotein and said composition contains said peptide in an amount sufficient to enhance the induction of persistent neutralizing antibodies in the host.

29. Composition as claimed in claim 28, wherein said at least one peptide comprises a mixture of peptides of glycoprotein of HIV.

30. Composition as claimed in claim 28, wherein the peptides are bound to a carrier therefor.

31. Composition as claimed in claim 28, wherein the composition contains an adjuvant in an amount sufficient to enhance vaccination of the host.

32. Composition as claimed in claim 31, wherein the adjuvant is muramyl dipeptide or incomplete Freund's adjuvant.

33. Composition as claimed in claim 32, wherein the peptide is selected from the group consisting of *env*, *pol*, *gag*, *nef*, *vif* antigen, and mixtures of said antigens.

34. Composition as claimed in claim 33, wherein said mixture of said antigens comprises p27*nef* and p23*vif*.

35. Composition as claimed in claim 33, wherein the peptide is at least one peptide selected from the group consisting of:

C-TRPNNNTRKR IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKK IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRGS IRIQRGPGRA FVTIGK-IGN M-RQAH-C
C-TRPNNNTRKS IYI--GPGRA FHTTGRIIGD -IRKAH-C
C-TRPYNNVRRS LSI--GPGRA FRTRE-IIGI -IRQAH-C
C-TRPGNNTRRG IHF--GPGQA LYTTGIV-GD -IRRAY-C
C-ARPYQNTRQR TPI--GLGQS LYTTRSR-SI -IGQAH-C
C-TRPNNNTRKS ITK--GPGRV IYATGQIIGD -IRKAH-C
C-TRPNNNTRKR ITM--GPGRV YYTTGQIIGD -IRRAH-C
C-TRPGSDKRQS TPI--GLGQA LYTTRGRTKI -IGQAH-C
C-TRPGSDKKIR QSIRIGPGKV FYAKGG---I -TGQAH-C
C-TRPNNNTKKG IAI--GPGRT LYAREKIIGD -IRQAH-C
C-TRPNNHTRKR VTL--GPGRV WYTTGEILGN -IRQAH-C
C-TRPGNNTRRG SHF--GPGQA LYTTGIVGDI -RRAY-C
C-TRPDNKITSRQ-TPI-GLGQA LYTTRIKGDI -RQAY-C
C-TRPNNNVRRR-HIHI-GPGRA FYTGEIRNI -RQAH-C
C-TRPYKNTRQS-TPI--GLGQA LYTTRTKSI -GQAH-C
C-TRPNNNTTRS-IHI--GPGRA FYATGDIIGTIRQAH-C
C-TRPNYNKRKR-IHI--GPGRA FYTTKNIIGDIRQAH-C

36. Composition as claimed in claim 33, wherein the peptide comprises the following amino acid sequence:

YNTRKSIRIQRGPGRAFVTIGKIGN.

37. Composition as claimed in claim 33, wherein said at least one peptide is comprised of the major neutralization epitope (loop V3) of at least one HIV-1 isolate.

38. A composition for enhancing the immunogenicity of an envelope glycoprotein or a fragment of a virus, wherein the composition comprises as a combined preparation for simultaneous, separate, or sequential use:

(A) at least one envelope glycoprotein of the virus or a fragment of at least 50 amino acids of the glycoprotein; and

(B) at least one peptide derived form the amino acid sequence of the envelope glycoprotein;

wherein the peptide comprises at least one virus-neutralization epitope;

and wherein the envelope glycoprotein and the peptide are administered in an amount sufficient to induce neutralizing antibodies in the host.

39. A composition for enhancing the immunogenicity of an envelope glycoprotein of a virus, wherein the composition comprises, as a combined preparation for simultaneous, separate, or sequential use:

(A) at least one envelope glycoprotein of the virus or a fragment of the glycoprotein having its immunogenic properties in an amount sufficient for priming the induction of neutralizing antibodies in a host to which the envelope glycoprotein is administered; and

(B) at least one peptide derived from the amino acid sequence of said envelope glycoprotein;

wherein the peptide comprises at least one virus-neutralization epitope of said glycoprotein and said composition contains said peptide in an amount sufficient to enhance the induction of persistent neutralizing antibodies in the host.

40. A composition as claimed in claim 39, wherein the envelope glycoprotein or a fragment is gp160 of HIV or gp120 of HIV.

41. Composition as claimed in claim 40, wherein said at least one peptide comprises a mixture of peptides of glycoprotein of HIV.

42. Composition as claimed in claim 41, wherein said at least one peptide is bound to a carrier molecule comprising an aliphatic sequence.

43. Composition as claimed in claim 42, wherein the peptide is selected from the group consisting of the *env*, *gag* and especially p18*gag*, *nef*, *vif*, *pol*, or GPG OR GLG antigens and mixtures of said antigens, particularly p27*nef* and p23*vif*.

44. Composition as claimed in claim 42, wherein the envelope glycoprotein of the virus is combined with at least one of the antigens selected from the group consisting of *gag, pol, nef, vif,* and particularly with a mixture of p27*nef* and p23*vif*.

45. Composition as claimed in claim 39, wherein the composition is suitable for oral, parenteral, or intradermal administration.

46. Composition as claimed in claim 39, wherein the envelope glycoprotein or a fragment thereof and the peptide(s) derived therefrom are presented side-by-side in order to be applied simultaneously, separately, or at intervals to the host.

47. Composition as claimed in claim 39, wherein the envelope glycoprotein is combined with a pharmaceutical vehicle for oral or parenteral administration.

48. Composition as claimed in claim 39, wherein the peptide is combined with a pharmaceutical vehicle for oral administration.

49. Composition as claimed in claim 39, wherein either said at least one envelope glycoprotein of the virus and/or said at least one peptide derived from the envelope glycoprotein are presented:

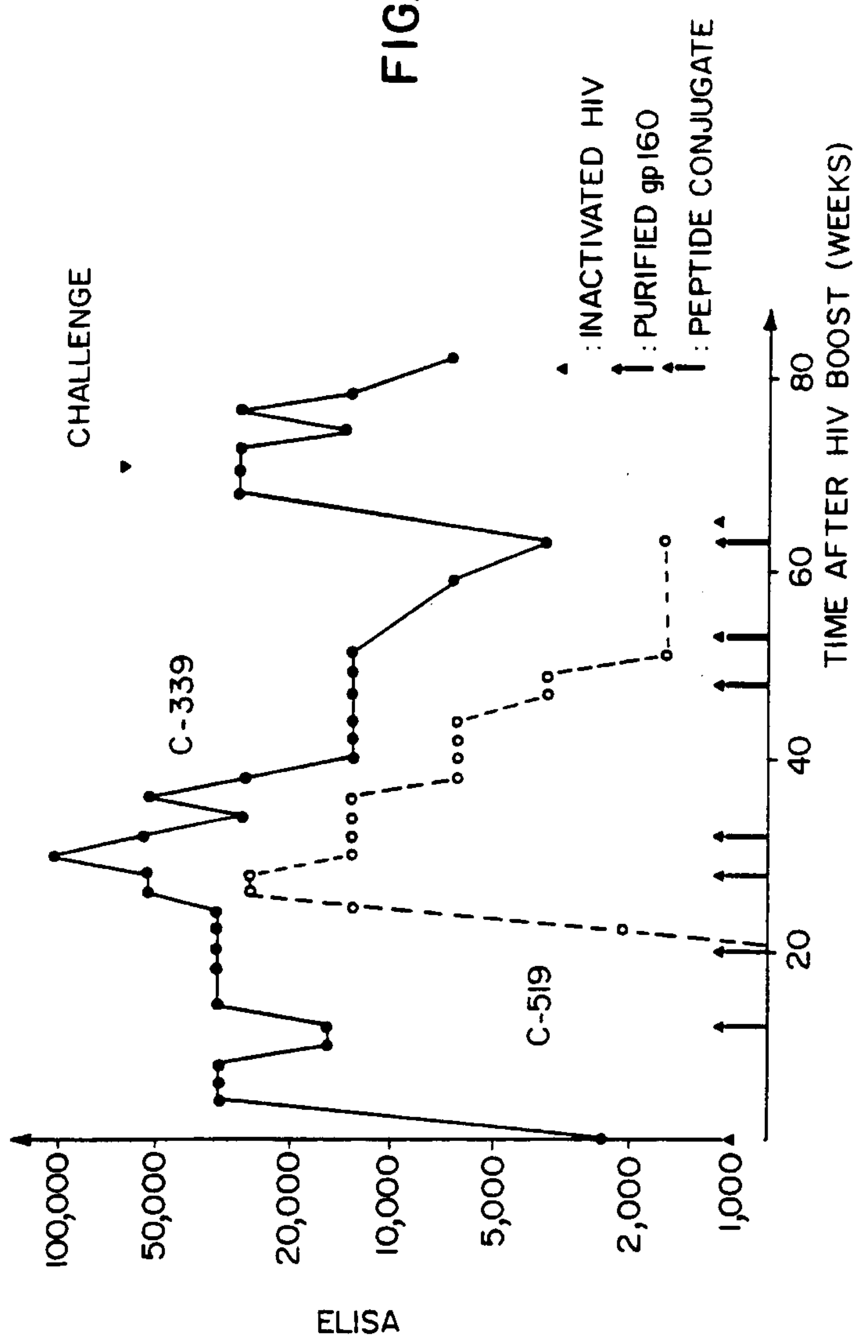
either under the form of particles, such as ISCOMs or liposomes,

or by a live recombinant microorganism.

50. Composition as claimed in claim 49, wherein the microorganisms is a live recombinant microorganism, such as viruses or bacteria, for instance a poxvirus or BCG, or any live vaccine modified to express the envelope glycoprotein or the peptide derived from the envelope glycoprotein.

51. Composition as claimed in claim 50, wherein the microorganism is derived from inactivated particles, for instance viral particles, such as the HIV virus, or particles without virus genome, especially without HIV genome.

FIG. 1


CHALLENGE
C-339
C-519
ELISA
100,000
50,000
20,000
10,000
5,000
2,000
1,000
20
40
60
80
TIME AFTER HIV BOOST (WEEKS)
: INACTIVATED HIV
: PURIFIED gp160
: PEPTIDE CONJUGATE

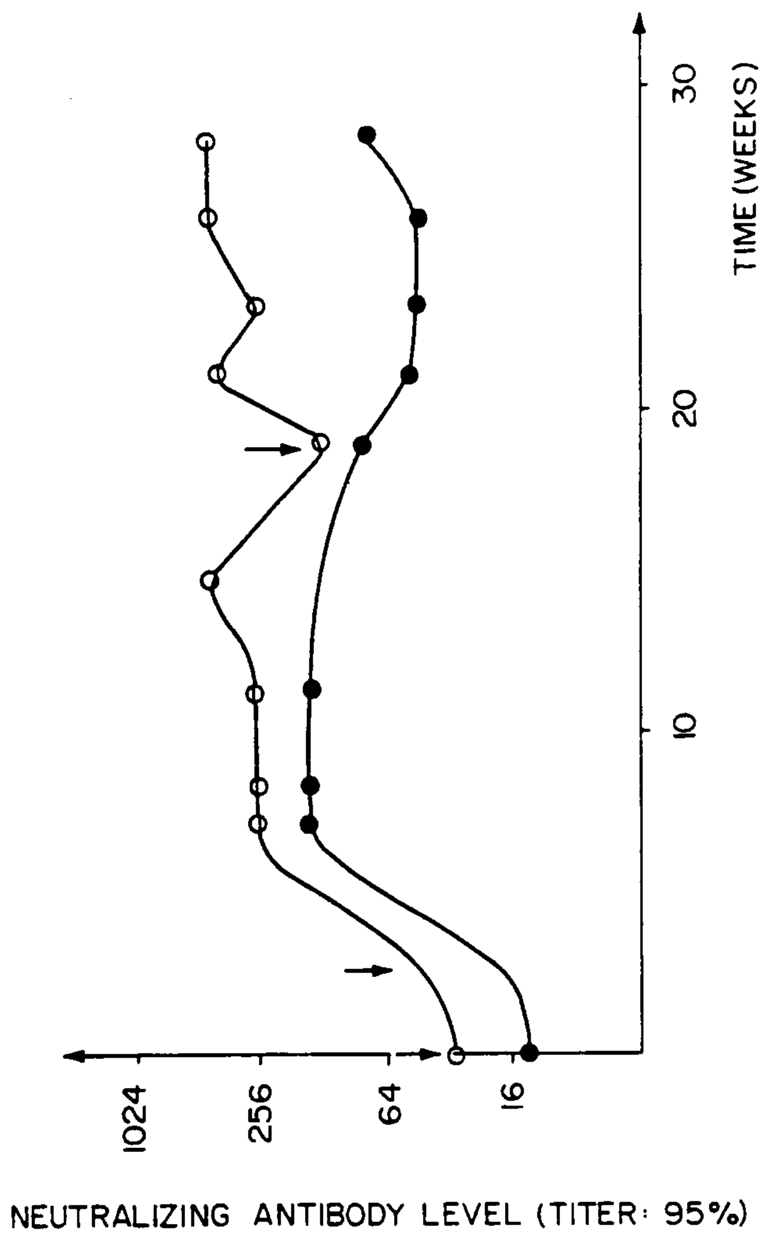
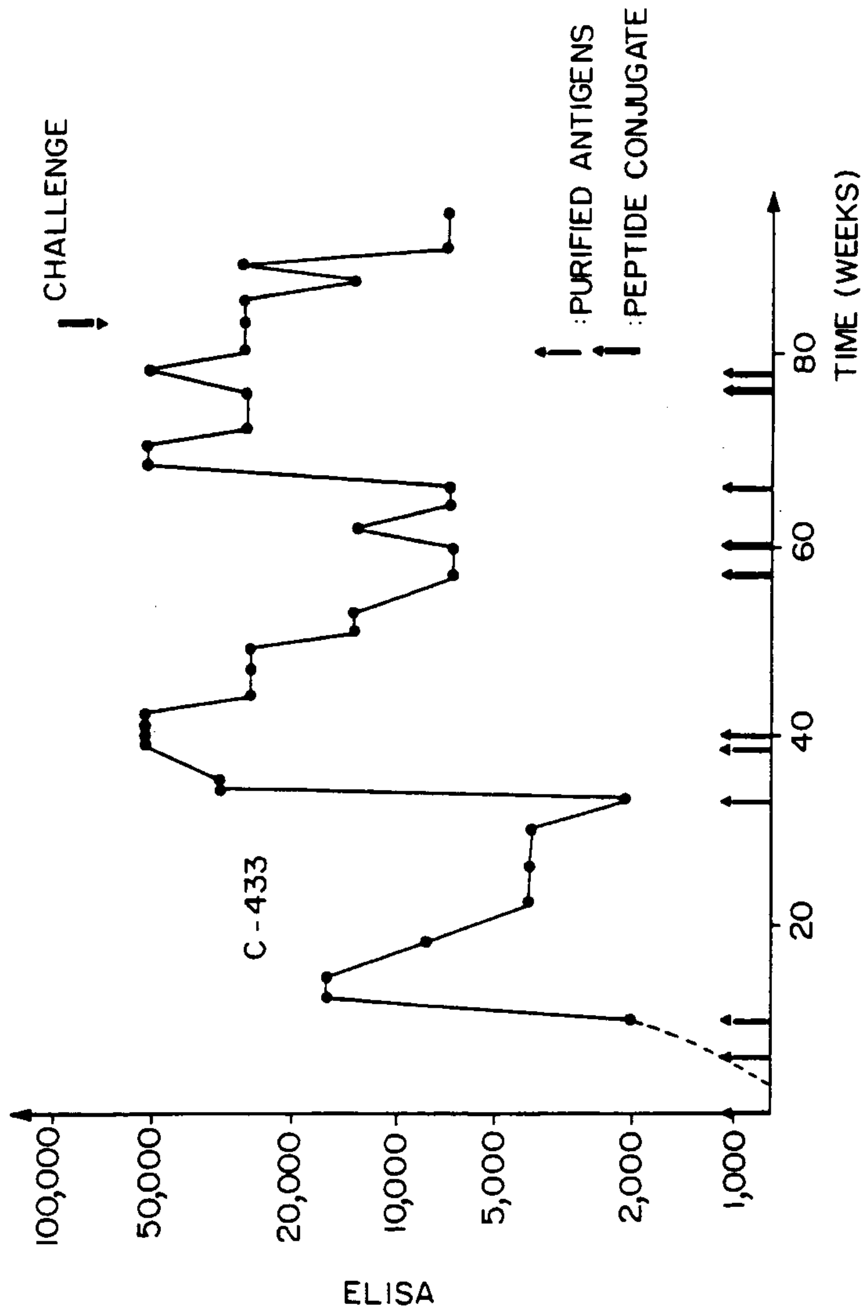

1024
256
64
16
10
20
30
TIME (WEEKS)
NEUTRALIZING ANTIBODY LEVEL (TITER: 95%)

FIG. 2

FIG. 3


CHALLENGE
:PURIFIED ANTIGENS
:PEPTIDE CONJUGATE
C - 433
ELISA
100,000
50,000
20,000
10,000
5,000
2,000
1,000
20
40
60
80
TIME (WEEKS)

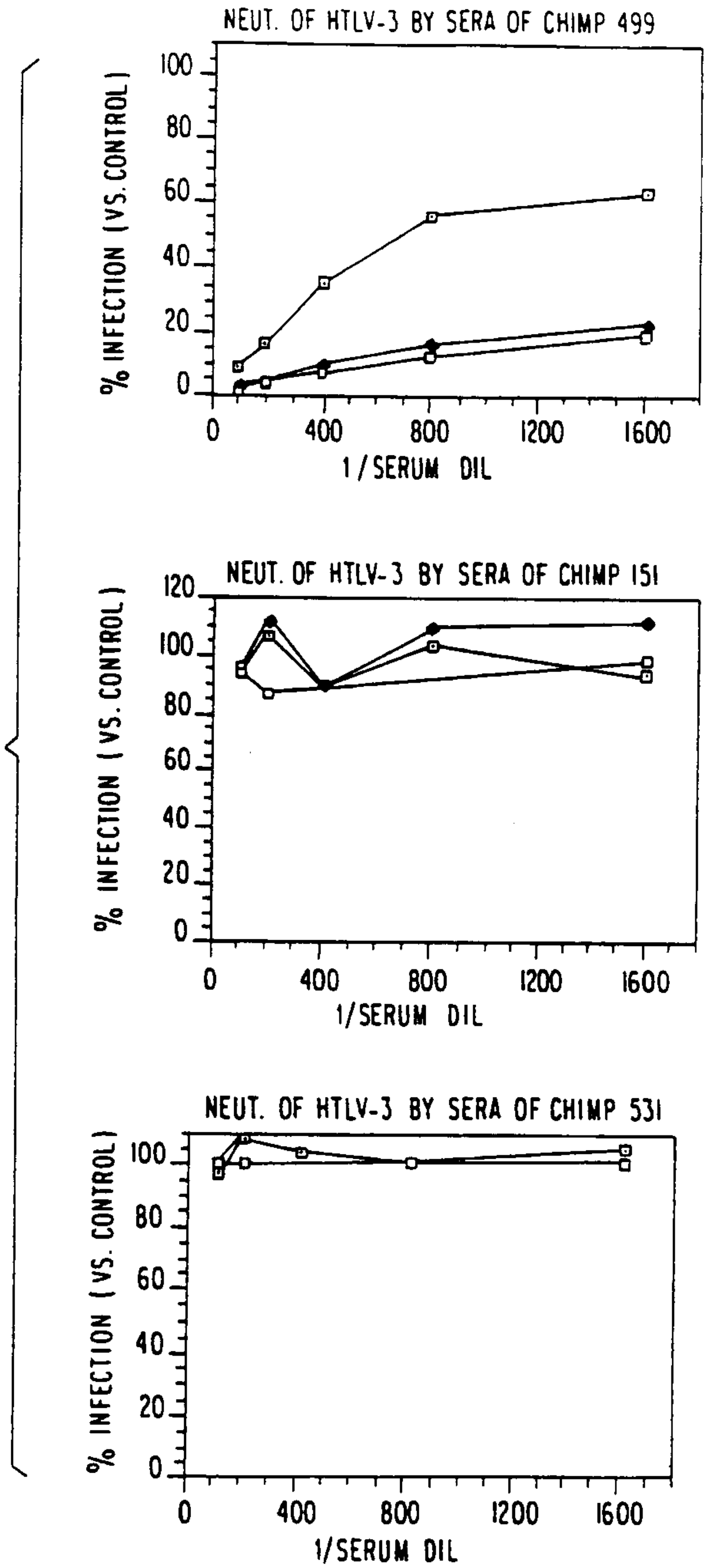
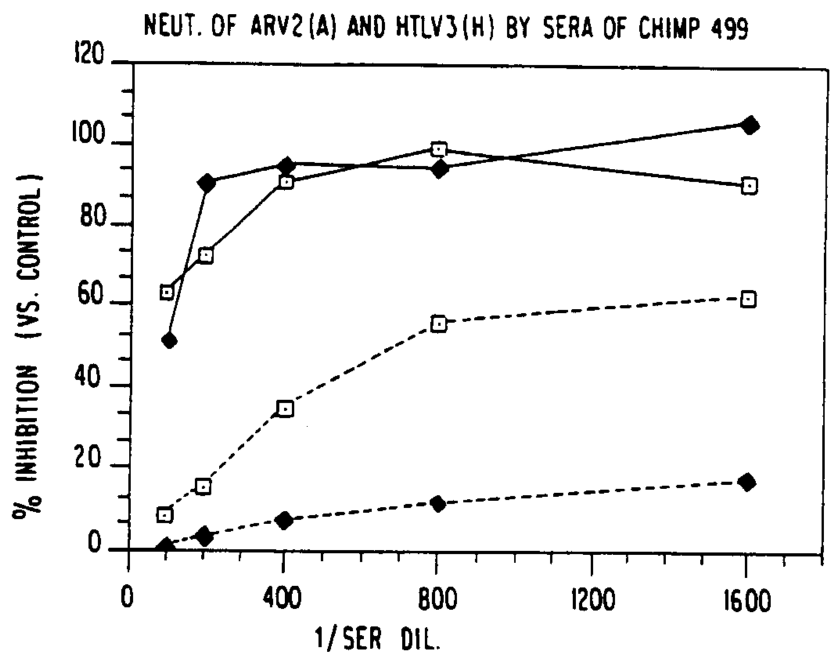

NEUT. OF HTLV-3 BY SERA OF CHIMP 499
% INFECTION (VS. CONTROL)
0
20
40
60
80
100
0
400
800
1200
1600
1 / SERUM DIL
NEUT. OF HTLV-3 BY SERA OF CHIMP 151
% INFECTION (VS. CONTROL)
0
20
40
60
80
100
120
0
400
800
1200
1600
1/SERUM DIL
NEUT. OF HTLV-3 BY SERA OF CHIMP 531
% INFECTION (VS. CONTROL)
0
20
40
60
80
100
0
400
800
1200
1600
1/SERUM DIL

FIG. 4

FIG. 5


NEUT. OF ARV2(A) AND HTLV3(H) BY SERA OF CHIMP 499
120
100
80
60
40
20
0
% INHIBITION (VS. CONTROL)
0
400
800
1200
1600
1/SER DIL.

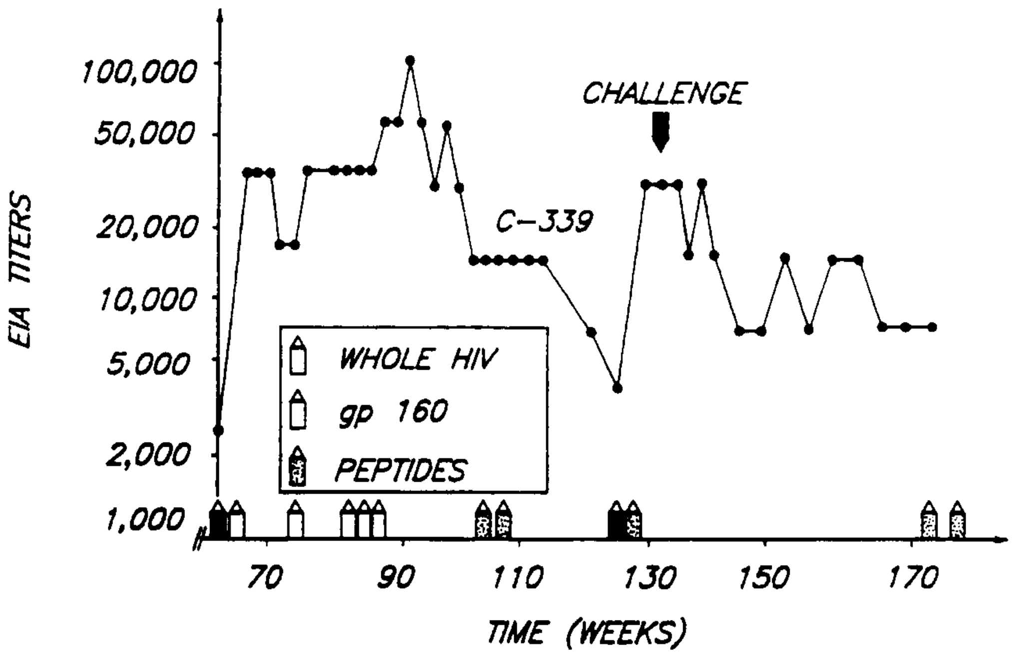

100,000
50,000
20,000
10,000
5,000
2,000
1,000
EIA TITERS
CHALLENGE
C-339
WHOLE HIV
gp 160
PEPTIDES
70
90
110
130
150
170
TIME (WEEKS)

FIG. 6A

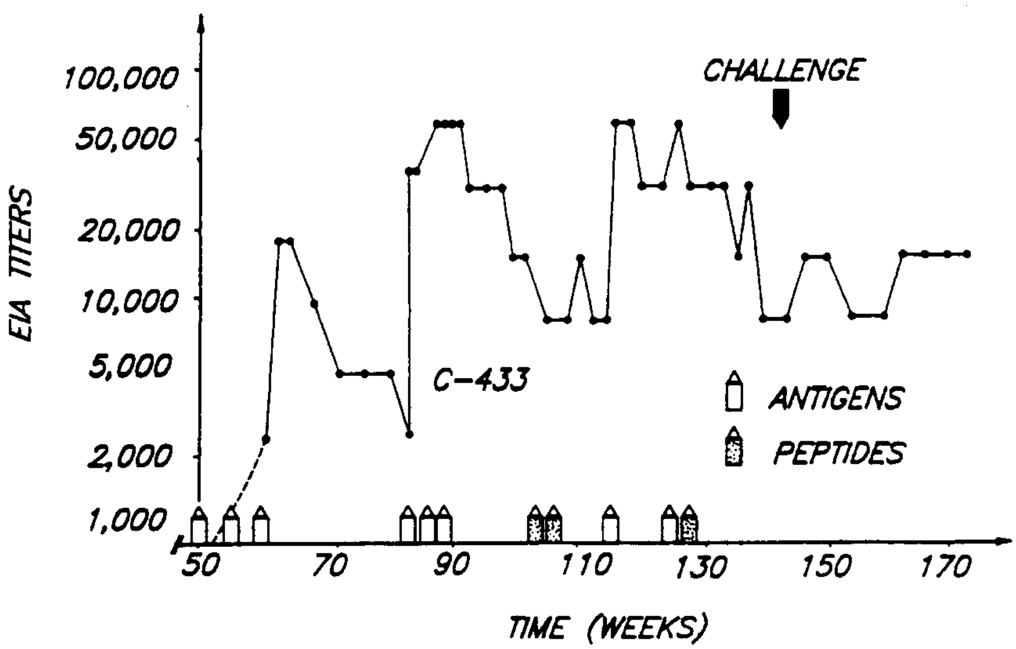
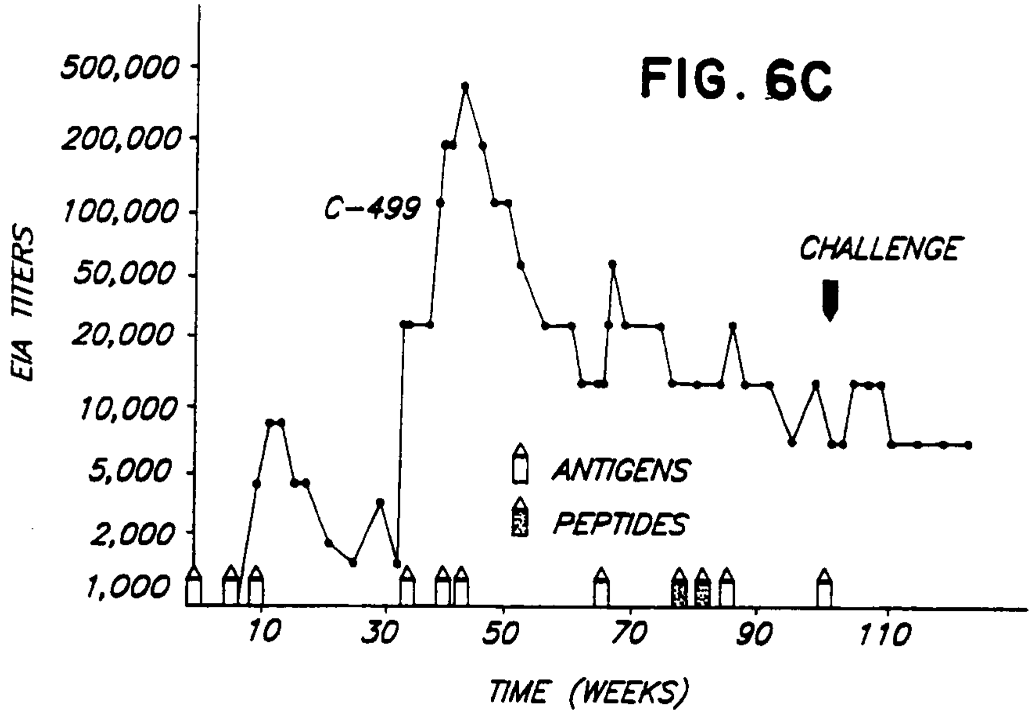
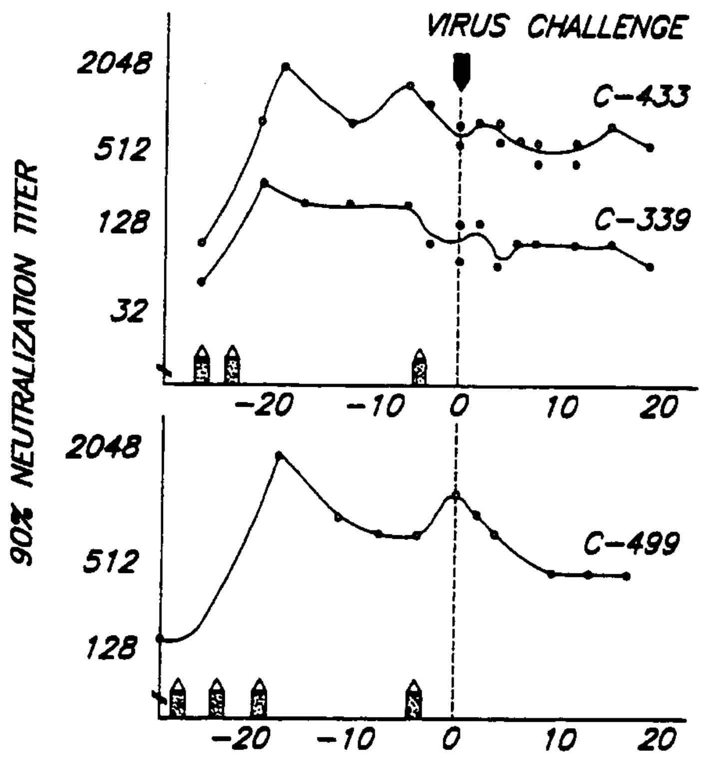

100,000
50,000
20,000
10,000
5,000
2,000
1,000
EIA TITERS
CHALLENGE
C-433
ANTIGENS
PEPTIDES
50
70
90
110
130
150
170
TIME (WEEKS)

FIG. 6B



500,000
200,000
100,000
50,000
20,000
10,000
5,000
2,000
1,000
EIA TITERS
C-499
CHALLENGE
ANTIGENS
PEPTIDES
10
30
50
70
90
110
TIME (WEEKS)

FIG. 6C



VIRUS CHALLENGE
2048
512
128
32
C-433
C-339
90% NEUTRALIZATION TITER
-20
-10
0
10
20
2048
512
128
C-499
TIME (WEEKS)

FIG. 7

FIG. 8A

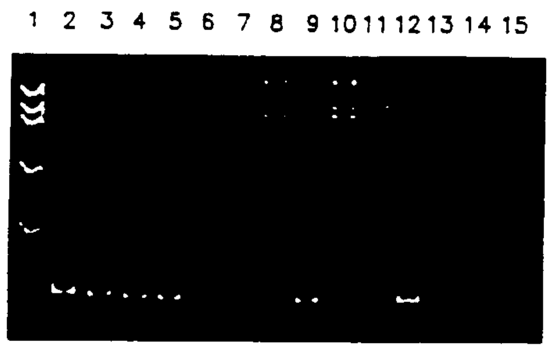


FIG. 8B

FIG. 8C

FIG. 9

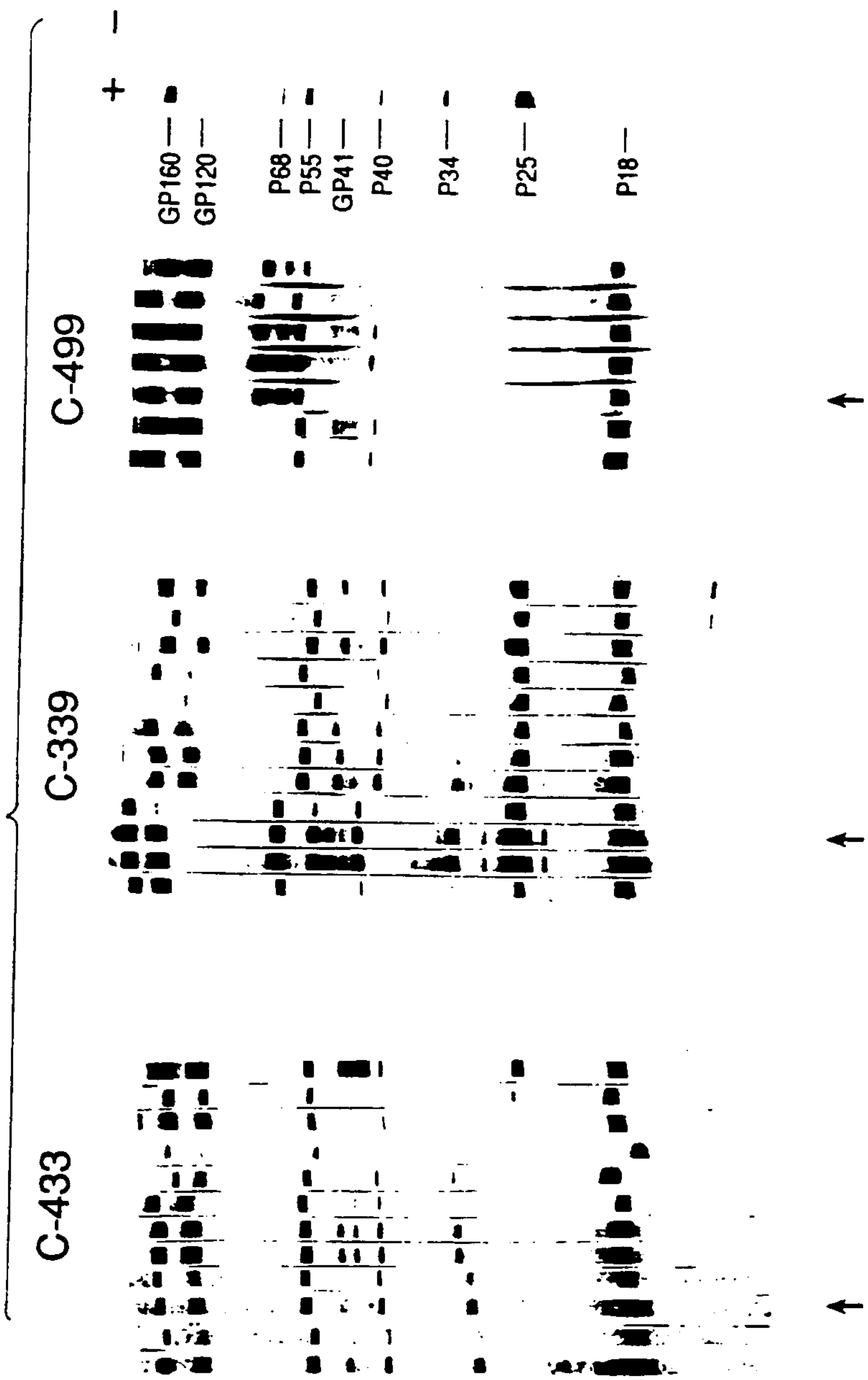


FIG. 10

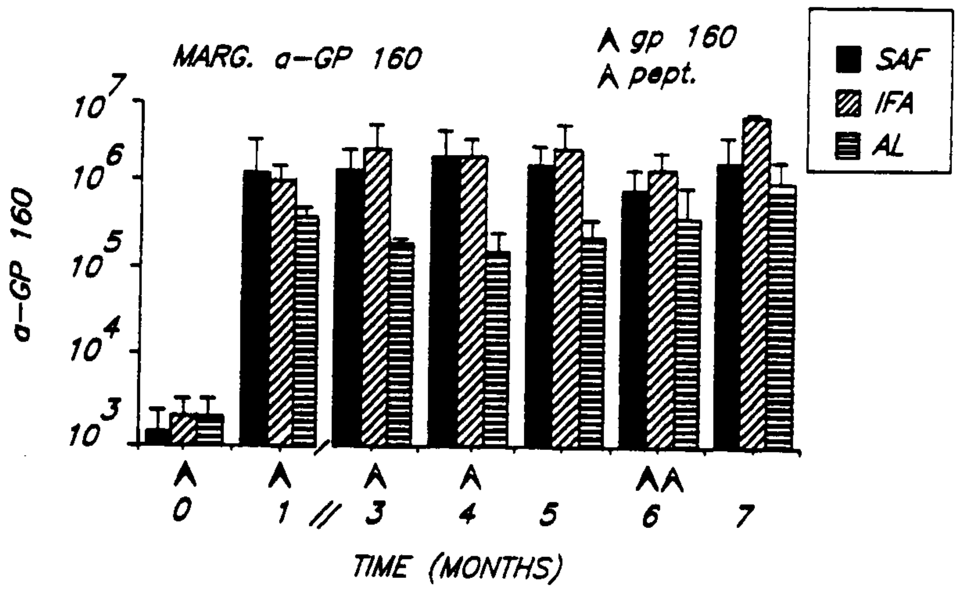
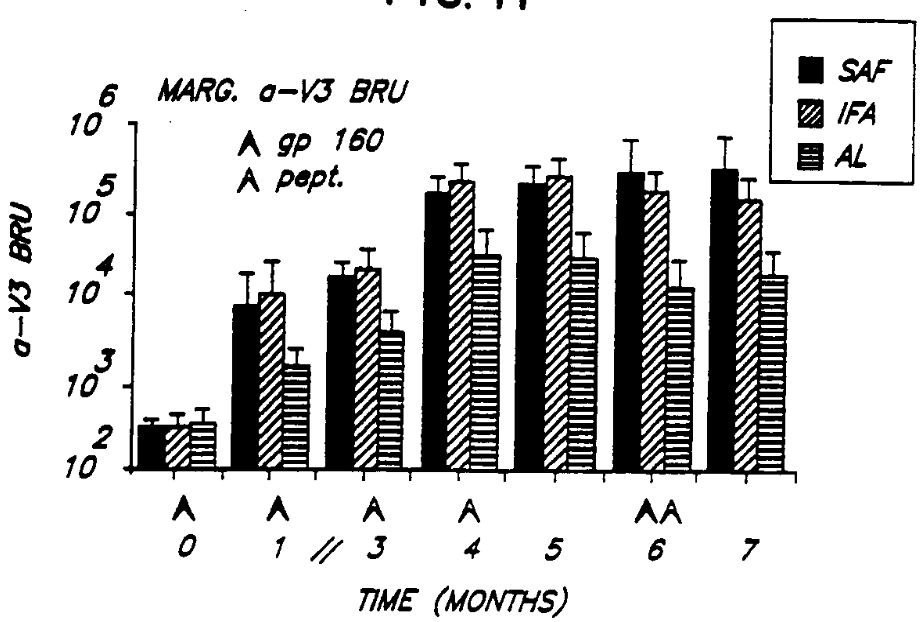

MARG. a-GP 160
gp 160
pept.
SAF
IFA
AL
10^7
10^6
10^5
10^4
10^3
a-GP 160
0
1
3
4
5
6
7
TIME (MONTHS)

FIG. 11


MARG. a-V3 BRU
gp 160
pept.
SAF
IFA
AL
10^6
10^5
10^4
10^3
10^2
a-V3 BRU
0
1
3
4
5
6
7
TIME (MONTHS)

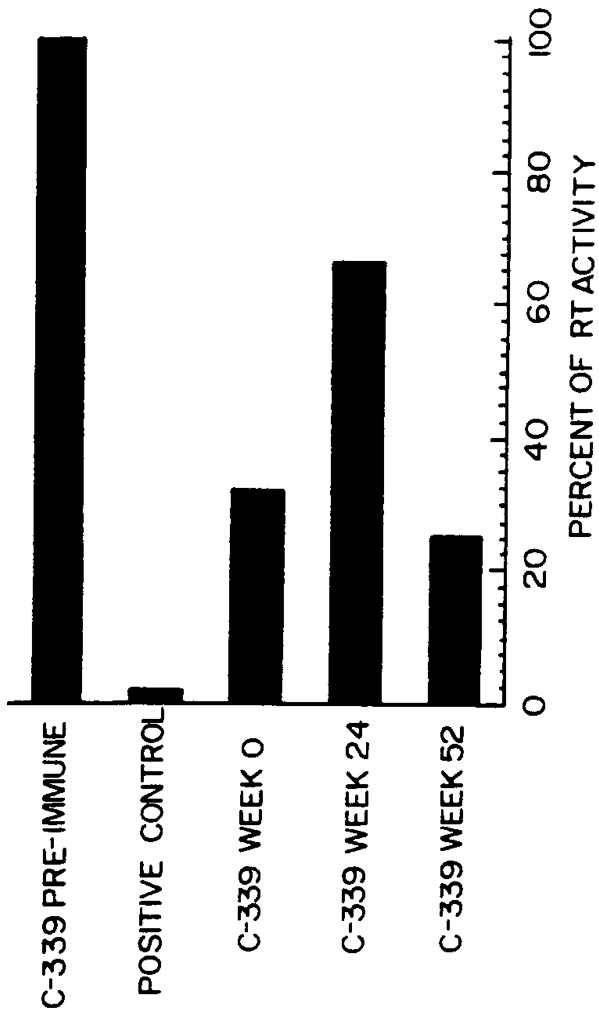
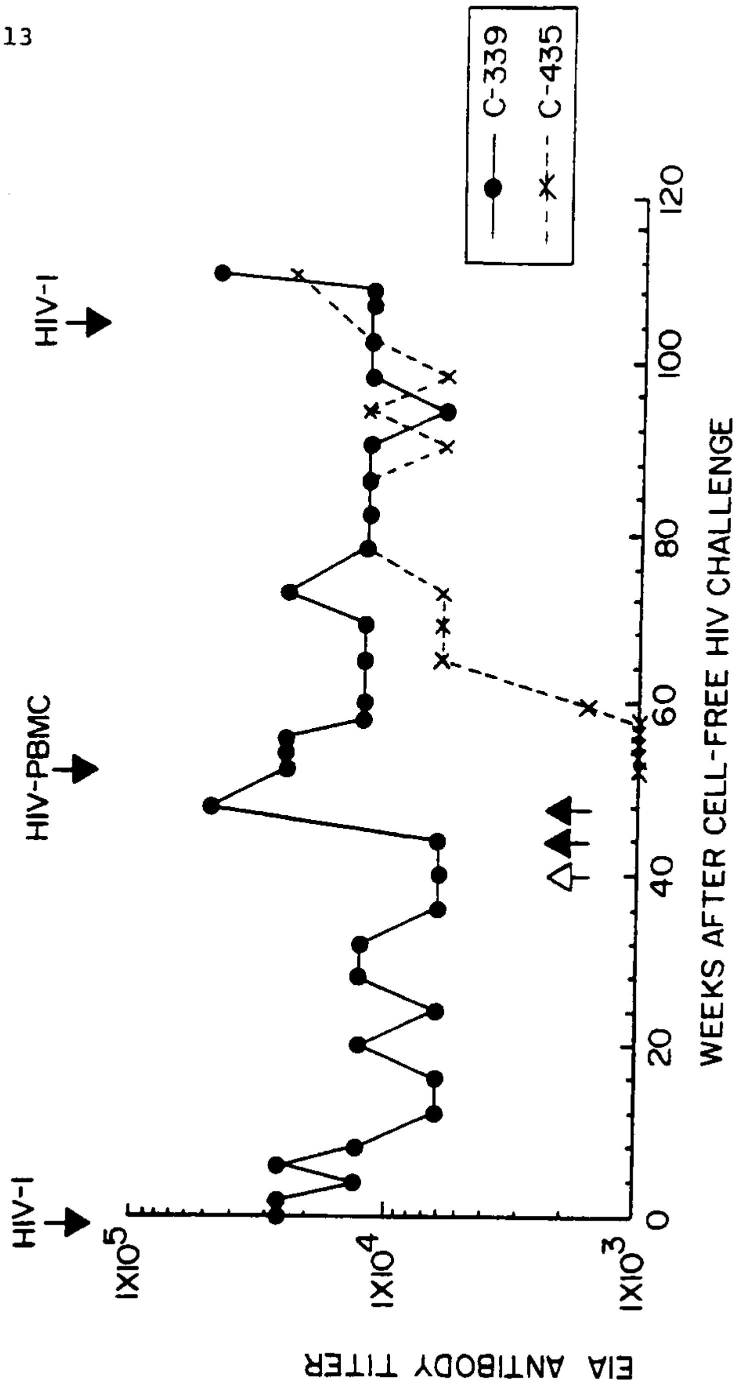

C-339 PRE-IMMUNE
POSITIVE CONTROL
C-339 WEEK 0
C-339 WEEK 24
C-339 WEEK 52
0
20
40
60
80
100
PERCENT OF RT ACTIVITY

FIG 12

FIG 13


HIV-I
HIV-PBMC
HIV-I
C-339
C-435
EIA ANTIBODY TITER
1X10^5
1X10^4
1X10^3
0
20
40
60
80
100
120
WEEKS AFTER CELL-FREE HIV CHALLENGE

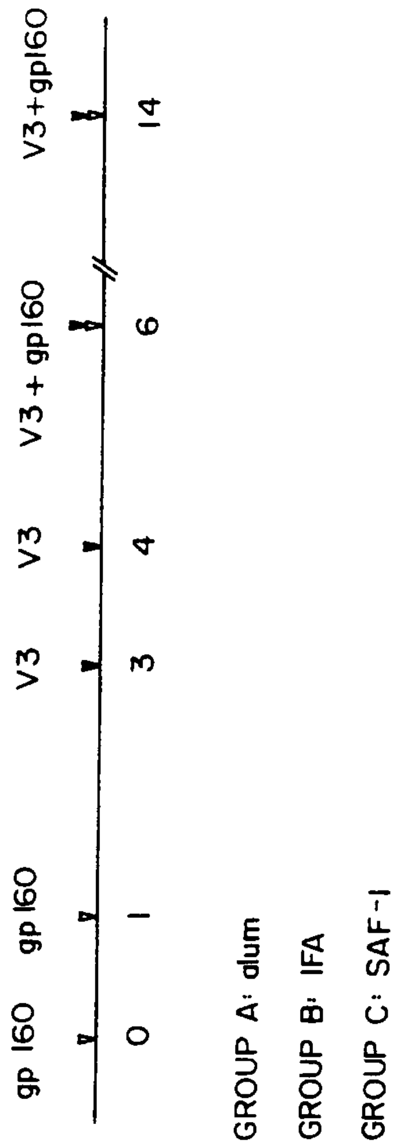

gp 160
gp 160
V3
V3
V3 + gp160
V3+gp160
0
1
3
4
6
14
GROUP A: alum
GROUP B: IFA
GROUP C: SAF-1

FIG 14

FIG 15

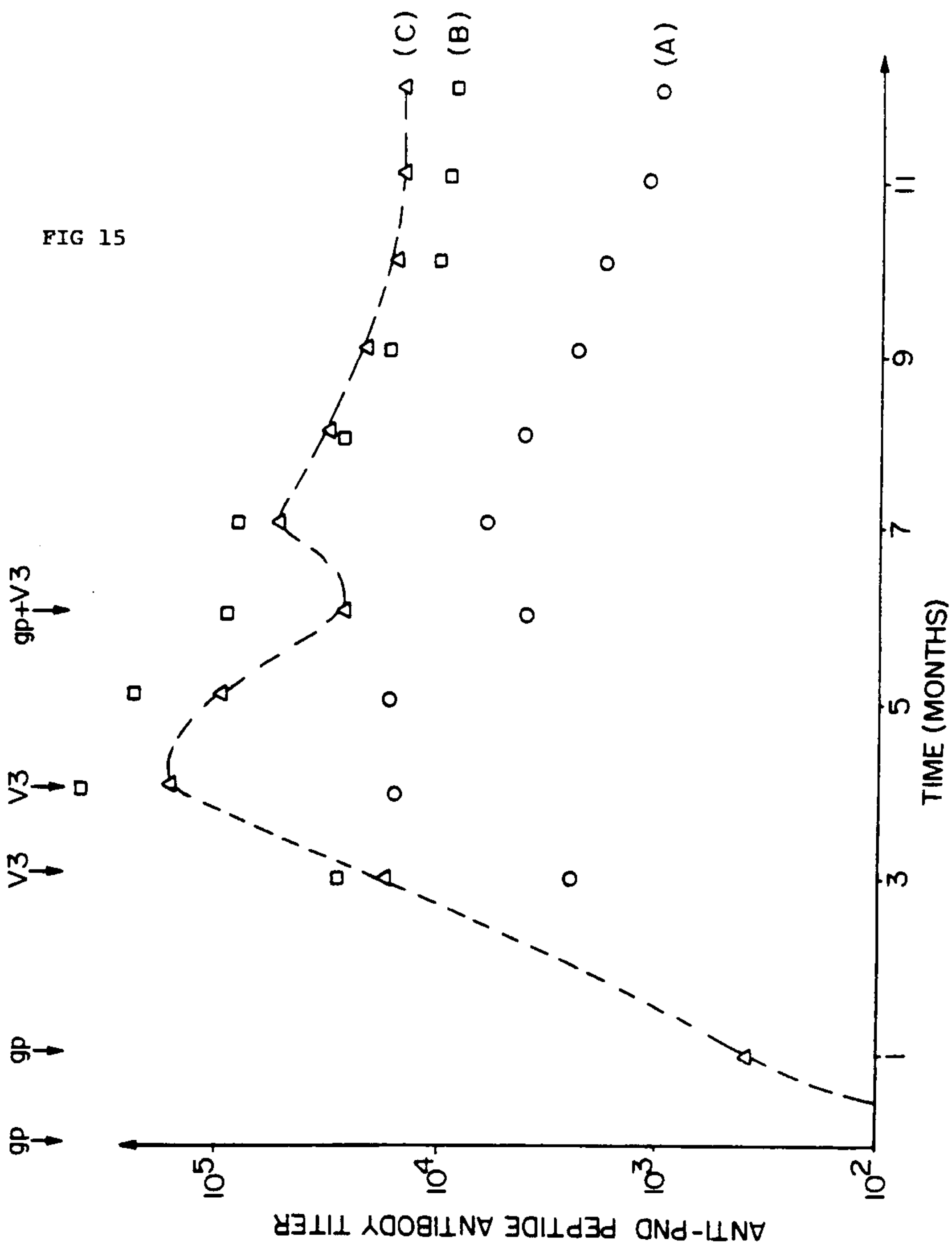
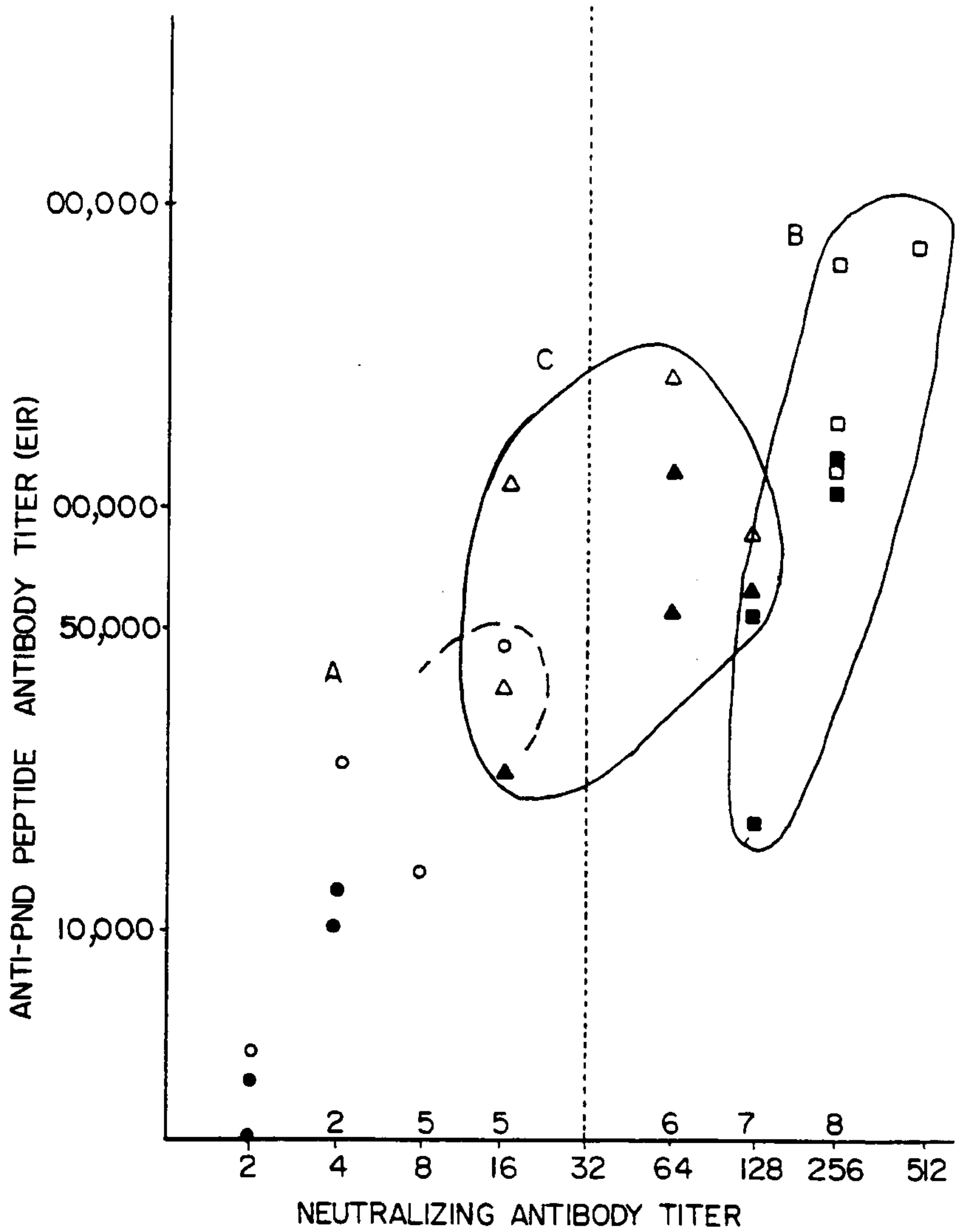

gp
gp
V3
V3
gp+V3
(C)
(B)
(A)
ANTI-PND PEPTIDE ANTIBODY TITER
10^2
10^3
10^4
10^5
TIME (MONTHS)
1
3
5
7
9
11

FIG 16



00,000
00,000
50,000
10,000
ANTI-PND PEPTIDE ANTIBODY TITER (EIR)
A
B
C
2
5
5
6
7
8
2
4
8
16
32
64
128
256
512
NEUTRALIZING ANTIBODY TITER

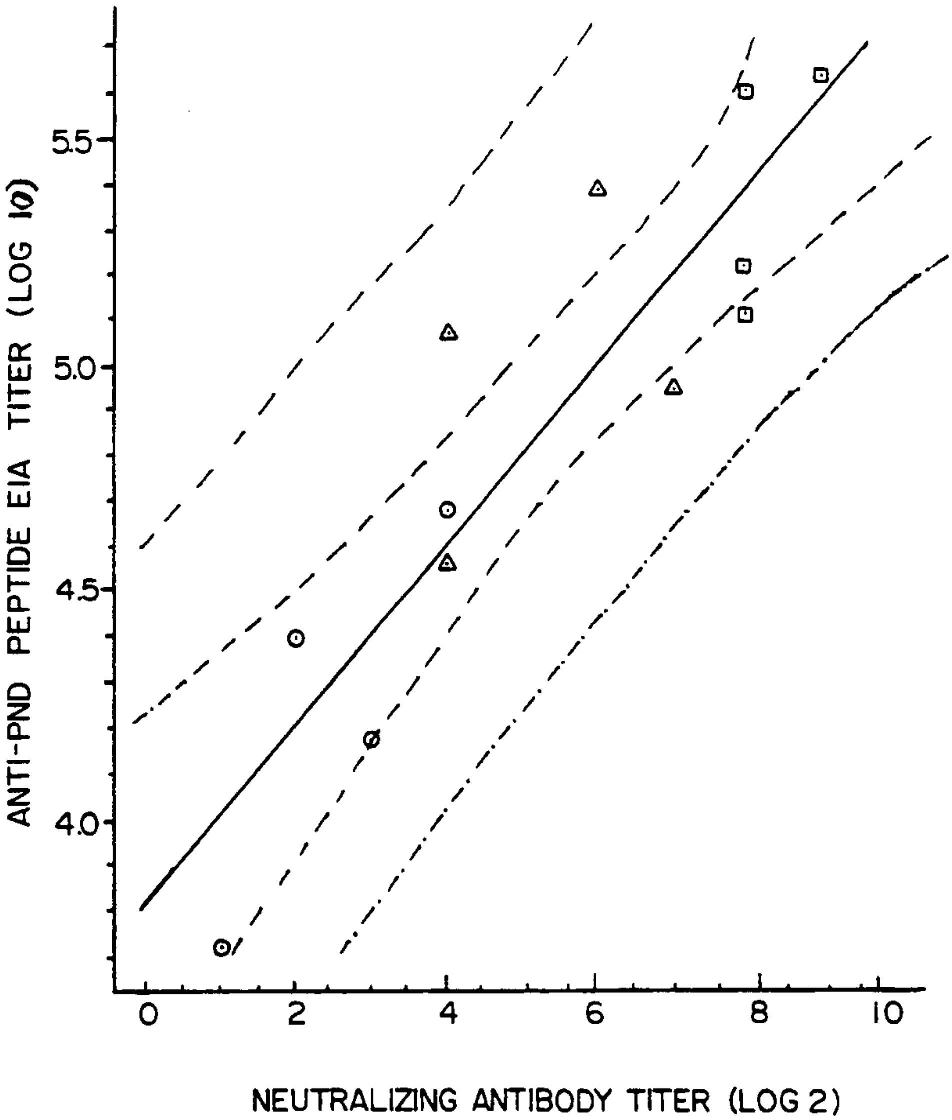
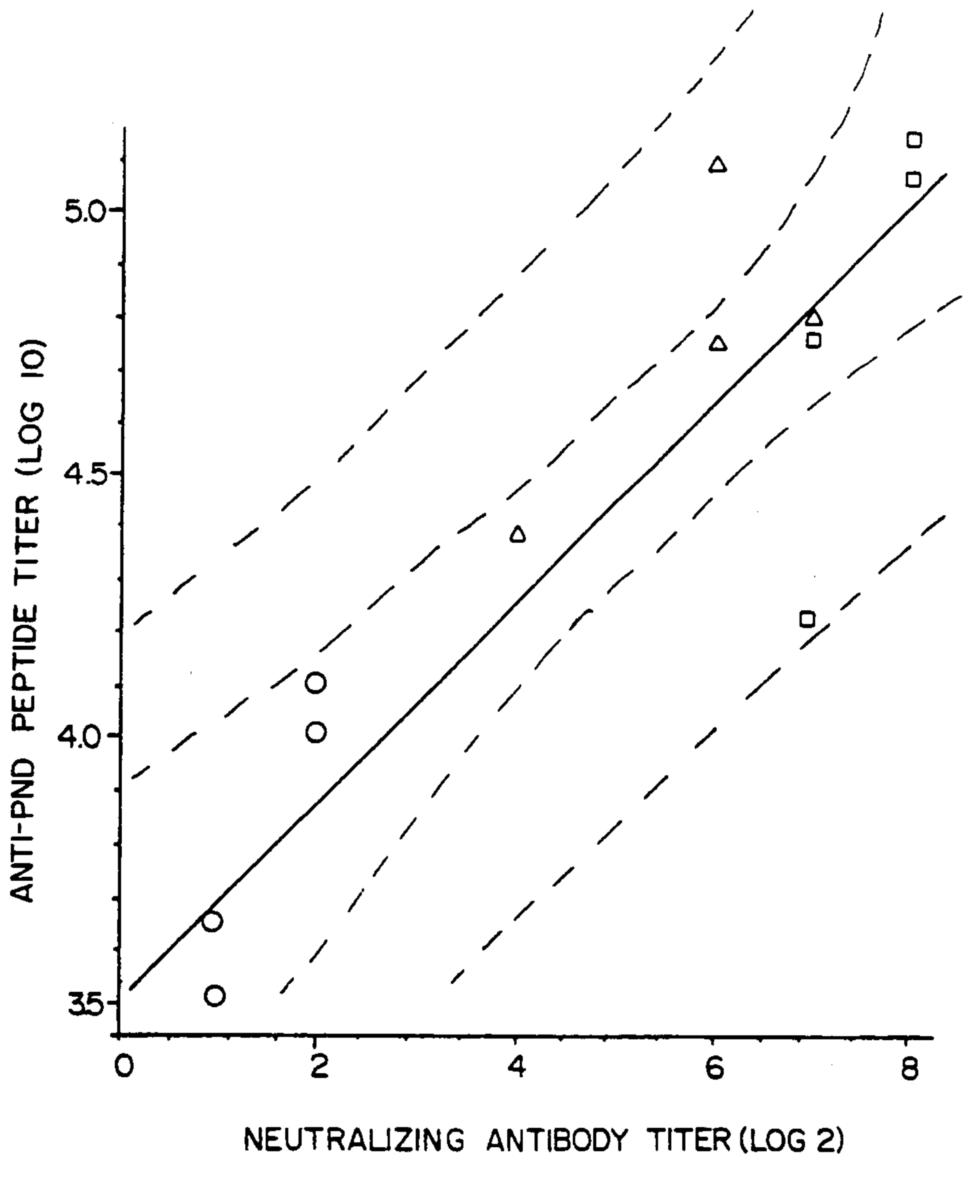

ANTI-PND PEPTIDE EIA TITER (LOG 10)
5.5
5.0
4.5
4.0
0
2
4
6
8
10
NEUTRALIZING ANTIBODY TITER (LOG 2)

FIG 17



5.0
4.5
4.0
3.5
0
2
4
6
8
ANTI-PND PEPTIDE TITER (LOG 10)
NEUTRALIZING ANTIBODY TITER (LOG 2)

FIG 18

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/02459

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K39/21

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 114 449 (INSTITUT PASTEUR) 3 October 1991 see the whole document -----	1-51

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 FEBRUARY 1993	05.03.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SITCH W.D.C.

1

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-27 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9202459
SA 66978

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/02/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9114449	03-10-91	AU-A- 7498991	21-10-91
		EP-A- 0472706	04-03-92
		JP-A- 3271233	03-12-91
		JP-T- 4506220	29-10-92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82